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Draft Cancer Human Health Hazard Assessment for Diisononyl Phthalate (DINP)

Technical Support Document for the Draft Risk Evaluation

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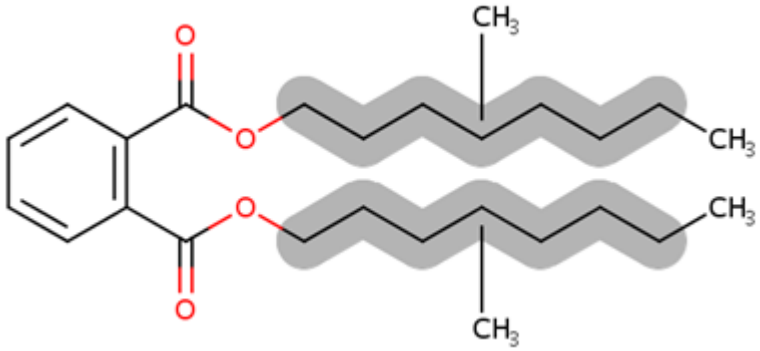

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May 2024

TABLE OF CONTENTS

LIST OF TABLES

LIST OF APPENDIX TABLES

ABBREVIATIONS AND ACRONYMS

α2u-globulin	Alpha 2u-globulin
AhR	Aryl hydrocarbon receptor
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
CAR	Constitutive androstane receptor
CASRN	Chemical abstracts service registry number
CPSC	Consumer Product Safety Commission (U.S.)
DINP	Diisononyl phthalate
DNA	Deoxyribonucleic acid
ECB	European Chemicals Bureau
ECHA	European Chemicals Agency
EFSA	European Food Safety Authority
EPA	Environmental Protection Agency (U.S.)
F344	Fischer 344 (rat)
GLP	Good Laboratory Practice
IARC	International Agency for Research on Cancer
KE	Key event
LOAEL	Lowest-observed-adverse-effect level
MNCL	Mononuclear cell leukemia
MOA	Mode of action
NF-κB	Nuclear factor kappa B
NICNAS	National Industrial Chemicals Notification and Assessment Scheme
NOAEL	No-observed-adverse-effect level
OCSPP	Office of Chemical Safety and Pollution Prevention
OEHHA	Office of Environmental Health Hazard Assessment (California)
OPPT	Office of Pollution Prevention and Toxics
POD	Point of departure
PPARα	Peroxisome proliferator-activated receptor alpha
PWG	Pathology Working Group
PXR	Pregnane X receptor
ROS	Reactive oxygen species
SACC	Science Advisory Committee on Chemicals
SD	Sprague-Dawley (rats)
TSCA	Toxic Substances Control Act
U.S.	United States

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As part of an intra-agency review, the draft DINP Risk Evaluation was provided to multiple EPA Program Offices for review. Comments were submitted by EPA's Office of Air and Radiation (OAR), Office of Children's Health Protection (OCHP), Office of General Counsel (OGC), ORD, and Office of Water (OW).

Docket

Supporting information can be found in the public docket, Docket ID (EPA-HQ-OPPT-2018-0436).

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1 INTRODUCTION

On May 24, 2019, the United States Environmental Protection Agency (U.S. EPA or the Agency) received a request, pursuant to 40 CFR 702.37, from ExxonMobil Chemical Company, through the American Chemistry Council's High Phthalates Panel (ACC HPP, 2019), to conduct a risk evaluation for diisononyl phthalate (DINP) (CASRNs 28553-12-0 and 68515-48-0) (Docket ID: EPA-HQ-OPPT-2018-0436). EPA determined that these two CASRNs should be treated as a category of chemical substances as defined in 15 U.S.C § 2625(c). On August 19, 2019, EPA opened a 45-day public comment period to gather information relevant to the requested risk evaluation. The Agency reviewed the request (along with additional information received during the public comment period) and assessed whether the circumstances identified in the request constitute conditions of use under 40 CFR 702.33, and whether those conditions of use warrant inclusion within the scope of a risk evaluation for DINP. EPA determined that the request meets the applicable regulatory criteria and requirements, as prescribed under 40 CFR 702.37. EPA granted the request on December 2, 2019, and published the draft and final scope documents for DINP in August 2020 and 2021, respectively (U.S. EPA, 2021, 2020).

Following publication of the final scope document, one of the next steps in the Toxic Substances Control Act (TSCA) risk evaluation process is to identify and characterize the human health hazards of DINP and conduct a dose-response assessment to determine the toxicity values to be used to estimate risks from DINP exposures. This technical support document summarizes the cancer hazards associated with exposure to DINP. Non-cancer hazards associated with exposure to DINP are summarized in a separate technical support document, the *Draft Non-cancer Human Health Hazard Assessment for Diisononyl Phthalate (DINP)* (U.S. EPA, 2024).

The carcinogenicity of DINP has been evaluated in existing assessments by Health Canada, U.S. Consumer Product Safety Commission (U.S. CPSC), European Chemicals Agency (ECHA), Australia National Industrial Chemicals Notification and Assessment Scheme (NICNAS), and California's Office of Environmental Health Hazard Assessment (OEHHA) (ECCC/HC, 2020; EC/HC, 2015; ECHA, 2013; Tomar et al., 2013; NICNAS, 2012; U.S. CPSC, 2010; ECB, 2003; U.S. CPSC, 2001). To date, DINP has been classified as a carcinogen by California OEHHA and is listed under California's Proposition 65 as a carcinogen (OEHHA, 2013; Tomar et al., 2013). Other authoritative agencies have not classified DINP as a carcinogen or evaluated DINP quantitatively for carcinogenic risk to human health.

This technical support document summarizes the available evidence for the carcinogenicity of DINP, the majority of which comes from experimental animal models. The remainder of this document is organized as follows:

- Section 2 summarizes available genotoxicity data for DINP.
- Section 3 summarizes available human and animal evidence for the carcinogenicity of DINP.
- Section 4 summarizes available liver tumor data and postulated mode of action (MOA) for liver tumors in rodents.
- Section 5 summarizes EPA's conclusions and next steps.
- Appendix A summarizes the results of a Pathology Working Group's review for spongiosis hepatis and mononuclear cell leukemia .

2 GENOTOXICITY AND MUTAGENICITY

The genotoxicity of DINP has been evaluated in several existing assessments, which have consistently concluded that DINP is not genotoxic nor is it likely to be genotoxic (ECCC/HC, 2020; EC/HC, 2015; ECHA, 2013; NICNAS, 2012; U.S. CPSC, 2010; EFSA, 2005; ECB, 2003; U.S. CPSC, 2001). EPA reviewed available genotoxicity studies of DINP that were cited in existing assessments (Table 2-1) and considered newer studies published between 2014 and 2019. No new genotoxicity studies of DINP were identified.

The mutagenic and genotoxic potential of DINP has been evaluated in 20 studies (Table 2-1). Available studies include two *in vivo* micronucleus tests in rodents, one *in vitro* chromosomal aberration assay, two *in vitro* mouse lymphoma assays, five bacterial reverse mutation assays, one *in vitro* unscheduled DNA synthesis assay, and nine *in vitro* cell transformation assays. No evidence of mutagenic activity was observed in five bacterial reverse mutation assays or two *in vitro* mouse lymphoma assays (with or without metabolic activation). DINP did not induce chromosomal aberrations in Chinese hamster ovary cells *in vitro*, cause unscheduled DNA synthesis in primary rat hepatocytes, or induce clastogenic effects or micronuclei formation *in vivo* in studies of mice or rats. Of the nine available *in vitro* transformation assays, only one study reported a positive result for transformation in Balb/c-3T3 A31 mouse cells in the absence of metabolic activation (Microbiological Associates, 1982c).

Consistent with the conclusions of existing assessments of DINP, available studies that evaluated the mutagenic and genotoxic potential of DINP are consistently negative. Therefore, EPA considers the weight of scientific evidence to indicate that DINP not likely to be genotoxic or mutagenic.

Table 2-1. Summary of Genotoxicity Studies of DINP

Test Type	Test System (Species/Strain/Sex)	Dose/Duration	Metabolic Activation	Result	Reference(s)
Chromosomal aberrations – *in vivo*					
Micronucleus (bone marrow) (Adhered to OECD 474)	Male CD-1 mice	Oral (gavage) doses of 0, 500, 1,000, or 2,000 mg/kg-day for 2 days; sacrificed on day 3	Not applicable	Negative for micronuclei	(McKee et al., 2000)
Chromosomal aberrations in femoral bone marrow cells	Male F344 rats	Oral (gavage) doses of 0, 0.5, 1.7, or 5.0 mL/kg-day for 5 days	Not applicable	Negative for micronuclei	(Microbiological Associates, 1982b)
Chromosomal aberrations – *in vitro*					
Chromosomal aberrations	Chinese hamster ovary cells	0, 40, 80, or 160 µg/mL for 3 hours (with activation) or 20 hours (without activation)	± Aroclor-induced rat liver S9	Negative for chromosomal aberrations	(McKee et al., 2000)
Gene mutations – *in vitro*					
Mouse lymphoma mutation assay	L5178Y+/- mouse lymphoma cells	0, 0.001, 0.01, 0.1, 1.0, 10, 100 µL/mL (±S9)	± Aroclor-induced rat liver S9	Negative for mutagenicity	(EG&G Mason Research Institute, 1982a)
Mouse lymphoma mutation assay	L5178Y+/- mouse lymphoma cells	1.5–8 µl/ml (−S9); 0.05–0.6 µL/mL (+ S9)	± Aroclor-induced rat liver S9	Negative for mutagenicity	(Barber et al., 2000)
Bacterial reverse mutation assay	*S. typhimurium* strains TA 98, TA 100,	0.1, 0.5, 2.5, 5, 10 µL/plate	± Aroclor-induced rat liver S9	Negative for mutagenicity	(EG&G Mason Research Institute, 1982b)

Test Type	Test System (Species/Strain/Sex)	Dose/Duration	Metabolic Activation	Result	Reference(s)
	TA 1535, TA 1537, TA 1538				
Bacterial reverse mutation assay	*S. typhimurium* strains TA 98, TA 100, TA 1535, TA 1537	0, 100, 333, 1,000, 3,333, 10,000 µg/plate	± Aroclor 1254- induced rat or hamster liver S9	Negative for mutagenicity	(Zeiger et al., 1985)
Bacterial reverse mutation assay	*S. typhimurium* strains TA 98, TA 100, TA 1535, TA 1537	20–5,000 µg/plate	± Aroclor-induced rat liver S9	Negative for mutagenicity	[(BASF, 1995, 1986) as reported by ECB (2003)][a]
Bacterial reverse mutation assay (plate incorporation assay)	*S. typhimurium* strains TA 98, TA 100, TA 1535, TA 1537, TA 1538	0.5–5,000 µg/plate	± Aroclor-induced rat liver S9	Negative for mutagenicity	(McKee et al., 2000)
Bacterial reverse mutation assay (pre-incubation assay)	*S. typhimurium* strains TA 98, TA 100, TA 1535, TA 1537	20–5,000 µg/plate	± Aroclor-induced rat liver S9	Negative for mutagenicity	(McKee et al., 2000)
Other genotoxicity assays					
Unscheduled DNA synthesis	Rat hepatocyte primary culture	0, 0.625, 1.25, 2.5, 5.0, 10.0 µL/mL	No	No increase in unscheduled DNA synthesis	(Litton Bionetics, 1982b)
In vitro cell transformation	Balb/c-3T3 A31 mouse cells	125–3,750 nL/mL	No	No significant increase in transformed foci	(Litton Bionetics, 1985)
In vitro cell transformation	Balb/c-3T3 A31 mouse cells	2.5–254.5 µg/mL	No	No significant increase in transformed foci	(Litton Bionetics, 1981)
In vitro cell transformation	Balb/c-3T3 A31 mouse cells	0.0326–3,260 µg/mL	No	No significant increase in transformed foci	(Litton Bionetics, 1982a)
In vitro cell transformation	Balb/c-3T3 A31 mouse cells	0.125–3.750 µL/mL	No	No significant increase in transformed foci	(Barber et al., 2000)
In vitro cell transformation	Balb/c-3T3 A31 mouse cells	0.1–1 µL/mL	± rat liver S9	No significant increase in transformed foci	(Microbiological Associates, 1982a)
In vitro cell transformation	Balb/c-3T3 A31 mouse cells	0.03–1 µL/mL	No	No significant increase in transformed foci	(Microbiological Associates, 1982c)
In vitro cell transformation	Balb/c-3T3 A31 mouse cells	0.01–1.0 µL/mL	No	No significant increase in	(Microbiological Associates, 1981)

Test Type	Test System (Species/Strain/Sex)	Dose/Duration	Metabolic Activation	Result	Reference(s)
				transformed foci	
In vitro cell transformation	Balb/c-3T3 A31 mouse cells	0.03–1 µL/mL	No	Positive (significant increase in transformed foci)	(Microbiological Associates, 1982d)
In vitro cell transformation	Balb/c- 3T3 mouse cells co-cultured with transformed cloned cells (strain 4-1-1)	5–5,000 ng/mL	No	No increase in proliferation rate of Balb/c 3T3 cells	(Fushiwaki et al., 2003)

[a] Study reports were not reasonably available to EPA. Information is as reported by ECB (2003).

234

3 CANCER HAZARD IDENTIFICATION AND CHARACTERIZATION

This section summarizes available human (Section 3.1) and animal evidence (Section 3.2) for the carcinogenicity of DINP. Section 3.2 discusses evidence for mononuclear cell leukemia (MNCL), kidney tumors, and other tumors observed in experimental animal models. Evidence for liver tumors in rodents and EPA's mode of action (MOA) analysis for liver tumors is provided in Section 4.

3.1 Human Evidence

No epidemiologic studies were identified by Health Canada (2018) that examined the association between DINP and its metabolites and biomarkers of cancer.

EPA identified two new medium quality studies that evaluated exposure to DINP and cancer. The first medium quality study, a case-control analysis by Parada et al., 2018 (2018) with a mortality follow-up component among women in the Long Island Breast Cancer Study Project, evaluated breast cancer mortality among cases with spot urine sample collected 3 months after breast cancer diagnosis. Inverse associations were observed between urine levels of two DINP metabolites (*i.e.,* MCNP and MCOP) and breast cancer for single quintiles, but the associations were not statistically significant.

The second medium quality study, a nested case-control study by Reeves et al. (2019) of the Women's Health Initiative prospective cohort, investigated the association between incident breast cancer cases in postmenopausal women and DINP. The authors found no significant association with one urinary DINP metabolite (*i.e.,* MCOP) and breast cancer in analysis using either ln-transformed or quartile exposure variables (adjusted odds ratio in models using ln-MCOP = 1.02; 95% CI: 0.90–1.16]). Findings were similar in models stratified by estrogen/progesterone receptor status and body mass index.

3.2 Animal Evidence

Four 2-year dietary studies evaluating the carcinogenicity of DINP in rodent models are available, including three studies of male and female Fischer 344 (F344) and Sprague-Dawley (SD) rats (Covance Labs, 1998b; Lington et al., 1997; Bio/dynamics, 1987) and one study of male and female B6C3F1 mice (Covance Labs, 1998a). Available studies have been discussed extensively in existing assessments of DINP. No new carcinogenicity studies of DINP with experimental laboratory animals were identified by EPA.

Across available studies, statistically significant increases in liver tumors, MNCL, and kidney tumors have been reported. Non-statistically significant increases in tumors in the testes, uterus, and pancreas have also been reported. Evidence for liver tumors, MNCL, kidney tumors, and other tumors is discussed in Sections 3.2.1 through 3.2.4.

3.2.1 Liver Tumors

The *Draft Non-cancer Human Health Hazard Assessment for Diisononyl Phthalate (DINP)* (U.S. EPA, 2024) describes the non-cancer liver effects observed following exposure to DINP in experimental animal models. Notably, many of the non-cancer liver effects observed in rodents following oral exposure to DINP comprise a suite of effects that may represent a progression from non-cancer to cancer (*e.g.,* increased liver weight, increased serum levels of ALT, AST, and ALP, histopathologic lesions such as hepatocellular hypertrophy and focal necrosis).

DINP has been evaluated for carcinogenicity in two 2-year dietary studies of F344 rats (Covance Labs, 1998b; Lington et al., 1997), one 2-year dietary study of SD rats (Bio/dynamics, 1987), and one 2-year

dietary study of B6C3F1 mice (Covance Labs, 1998a). Statistically significant increased incidences of tumors in the liver were reported in three out of four of the chronic 2-year studies (see Table 3-1 through Table 3-4). In one study, no statistically significant increases in neoplastic nodules and/or hepatocellular carcinomas were observed in male or female F344 rats treated with up to 307 to 375 mg/kg-day DINP for two-years (Table 3-1)—although hepatocellular cancer was observed in 3 out of 80 males from the high-dose groups compared to 0 out of 80 in controls (Lington et al., 1997; Bio/dynamics, 1986).

Two other studies of F344 and SD rats by Covance Labs (1998b) and Bio/dynamics (1987), respectively, included higher doses than Lington et al. (1997), and reported significant increases in hepatocellular adenoma and/or carcinoma (Table 3-2 and Table 3-3). Increased incidence of hepatocellular carcinoma (males only), and adenomas or carcinomas combined (both sexes) were observed in male and female F344 rats treated with up to 733 to 885 mg/kg-day DINP for 2 years (Covance Labs, 1998b) (Table 3-2). In the second study, hepatocellular carcinomas were significantly increased in high-dose female SD rats treated with 672 mg/kg-day DINP for 2 years, while no significant increase in neoplastic nodules or hepatocellular carcinomas were observed in male SD rats treated with up to 553 mg/kg-day DINP for 2 years (Table 3-3) (Bio/dynamics, 1987).

Finally, in a 2-year chronic study of DINP with B6C3F1 mice, the incidence of carcinomas was significantly increased in males at 1,560 mg/kg-day and females at 910 mg/kg-day and above, while the combined incidence of hepatocellular adenomas and carcinomas were significantly increased in both males (≥742 mg/kg-day) and females (≥336 mg/kg-day) (Table 3-4) (Covance Labs, 1998a).

3.2.1.1 Conclusions on Liver Tumors

Collectively, available studies provide consistent evidence that chronic oral exposure to DINP can cause treatment-related liver tumors in both sexes of several strains of rats (*i.e.,* F344 and SD) and mice (B6C3F1). EPA further considers the weight of evidence for liver carcinogenesis and its underlying MOA in Section 4.

Table 3-1. Incidences of Neoplastic Lesions in the Livers of Male and Female F344 Rats Exposed to DINP for 24 Months (Lington et al., 1997; Bio/dynamics, 1986)

Lesion	Dose Group mg/kg-day (ppm)			
	Control	15 M / 18 F (300)	152 M / 184 F (3,000)	307 M / 375 F (6,000)
Males[a]				
Neoplastic nodules	3/81 (3.7%)	1/80 (1.3%)	1/80 (1.3%)	1/80 (1.3%)
Hepatocellular cancer	0/81 (0%)	0/80 (0%)	0/80 (0%)	3/80 (3.8%)
Neoplastic nodules or cancer (combined)	3/81(3.7%)	1/80 (1.3%)	1/80 (1.3%)	4/80 (5.0%)
Females[a]				
Neoplastic nodules	0/80 (0%)	2/81 (2.5%)	0/80 (0%)	1/80 (1.3%)
Hepatocellular cancer	1/81 (1.2%)	0/81 (0%)	0/80 (0%)	1/80 (1.3%)
Neoplastic nodules or cancer (combined)	1/81 (1.2%)	2/81 (2.5%)	0/80 (0%)	2/80 (2.5%)

Source: Table 8 in Lington et al. (1997)
M = male; F = female
[a] Number of animals with lesion/ total number of animals examined. Percent lesion incidence in parentheses. No statistically significant increases in hepatocellular nodules and/or cancer was observed in either sex.

Table 3-2. Incidence of Liver Tumors in Male and Female F344 Rats Exposed to DINP in the Diet for 2 Years (Covance Labs, 1998b)[a] [b]

Lesion	Dose Group mg/kg-day (ppm)				
	Control	29 M / 36 F (500)	88 M / 109 F (1,500)	359 M / 442 F (600)	733 M / 885 F (12,000)
Males					
Hepatocellular adenoma	4/65[b] (6%)	3/50 (6%)	2/50 (4%)	6/65 (9%)	6/65 (15%)
Hepatocellular carcinoma	1/65 (2%)	0/50 (0%)	0/50 (0%)	1/65 (2%)	12/65* (18%)
Adenoma or carcinoma (combined)	5/65 (8%)	3/50 (6%)	2/50 (4%)	7/65 (11%)	18/65* (28%)
Females					
Hepatocellular adenoma	0/65 (0%)	1/49 (2%)	0/50 (0%)	1/65 (2%)	3/65 (5%)
Hepatocellular carcinoma	1/65 (2%)	0/49 (0%)	0/50 (0%)	1/65 (2%)	5/65 (8%)
Adenoma or carcinoma (combined)	1/65 (2%)	1/49 (2%)	0/50 (0%)	2/65 (3%)	8/65* (12%)

Source: U.S. CPSC (2001); Table IX-1 (pg. 68); text pages 68–71 and Appendix B.
M = male; F = female
* = statistically significant at $p < 0.05$ by one or more of the following: Fisher's Exact test, Poly-3, Logistic Regression, or Life Table analysis.
[a] Where results are of borderline significance or greater, level of statistical significance computed by logistic regression is given. Significance value for trend is given in the column for the control group. Significance values for these findings calculated using different statistical tests are given in Appendix B, section A. Analysis of individual animal data as performed by the National Toxicology Program (U.S. CPSC, 2001).
[b] Number of animals with neoplasm/ total number of animals examined. Percent tumor incidence in parentheses. Based on extraction and analysis of individual animal data as reported in U.S. CPSC (2001). Overall incidence for control, 6,000 ppm and 12,000 ppm groups (n = 65) includes incidence data for unscheduled deaths, interim sacrifice at week 78 and terminal sacrifice. Overall incidence for the remaining groups includes incidence data for unscheduled deaths and terminal sacrifice.

Table 3-3. Incidence of Neoplastic Lesions in the Liver of Male and Female SD Rats Exposed to DINP in the Diet for 2 Years (Bio/dynamics, 1987)[a]

Lesion	Dose Group mg/kg-day (ppm)			
	Control	27 M / 33 F (500)	271 M / 331 F (5,000)	553 M / 672 F (10,000)
Males				
Hepatocellular carcinoma	2/70 (2.9%)	2/69 (2.9%)	6/69 (8.7%)	4/70 (5.7%)
Neoplastic nodule(s)[b]	2/70 (2.9%)	5/69 (7.2%)	6/69 (8.7%)	5/70 (7.1%)
Females				
Hepatocellular carcinoma	0/70 (0%)†	0/70 (0%)	5/70 (7.1%)	7/70 (10%)*
Neoplastic nodule(s)	1/70 (1.4%)	1/70 (1.4%)	5/70 (7.1%)	2/70 (2.9%)

Source: Appendix K, Figure 1, pp. 11 (pp. 426 of the study report PDF) (Bio/dynamics, 1987).
* Statistically significant ($p \leq 0.05$) from the control group by a two-tailed Fisher's exact test
† Statistically significant trend ($p < 0.05$) based on a Chi-square contingency trend test calculated for this review.
[a] Data in this table indicate all animals assessed for histopathology throughout the study; *i.e.*, including the interim sacrifice, the terminal sacrifice, and unscheduled deaths. For late-developing tumors (hepatocellular carcinoma, pancreatic islet cell tumors, testicular interstitial cell tumors), statistical analysis was performed excluding animals that died or were sacrificed up to 12 months, leaving n = 57, 57, 59, 59 in males and n = 59, 56, 60, 59 in females in the control, low-, mid- and high-dose groups, respectively.
[b] Pathology report does not define this lesion further, which is a reporting deficiency that reduces the ability to compare results of Bio/dynamics (1987) to those of other studies which report incidences of hepatocellular adenomas, carcinomas, and adenomas or carcinomas, combined.

Table 3-4. Incidence of Liver Tumors in Male and Female B6C3F1 Mice Exposed to DINP in the Diet for 2 Years (Covance Labs, 1998a)

Lesion	Dose Group mg/kg-day (ppm)				
	Control	90 M / 112 F (500)	276 M / 336 F (1,500)	742 M / 910 F (600)	1,560 M / 1,888 F (12,000)
Males					
Hepatocellular adenoma	10/70[b] (14%)	7/67 (10%)	8/66 (12%)	15/65 (23%)	13/70 (19%)
Hepatocellular carcinoma	10/70 (14%)	8/67 (12%)	10/66 (15%)	17/65 (26%)	20/70* (29%)
Adenoma or carcinoma (combined)	16/70 (23%)	13/67 (19%)	18/66 (27%)	28/65* (43%)	31/70* (44%)
Females					
Hepatocellular adenoma	2/70 (3%)	4/68 (6%)	5/68 (7%)	4/67 (6%)	18/70* (26%)
Hepatocellular carcinoma	1/70 (1%)	2/68 (3%)	5/68 (7%)	7/67* (10%)	19/70* (27%)
Adenoma or carcinoma (combined)	3/70 (4%)	5/68 (7%)	10/68* (15%)	11/67* (16%)	33/70* (47%)

Source: U.S. CPSC (2001) Table IX-6 (page 73) and Appendix B.
M = male; F = female
* = significant from the control at $p < 0.05$ by logistic regression analysis
[a] Where results are of borderline significance or greater, level of statistical significance computed by logistic regression is given. Significance value for trend is given in the column for the control group. Significance values for these findings calculated using different statistical tests are given in Appendix B, section B (U.S. CPSC, 2001).
[b] Number of animals with tumor/total number of animals examined. Percent tumor incidence in parentheses.

3.2.2 Mononuclear Cell Leukemia

MNCL has been observed in F344 rats in two 2-year dietary studies (Covance Labs, 1998b; Lington et al., 1997; Bio/dynamics, 1986). In contrast, MNCL has not been observed in SD rats in a 104 week study (Bio/dynamics, 1987) nor in B6C3F1 mice exposed to DINP for at least 104 weeks (Covance Labs, 1998a).

Lington et al. (1997) reported the incidence data for MNCL. The incidence of MNCL was statistically significantly increased in the mid- and high-dose groups for both sexes when compared with the concurrent control groups (Table 3-5). MNCL was detected in 41, 35, 60, and 64 percent of males and 27, 25, 38, and 54 percent of females in the control, low-, mid-, and high-dose groups, respectively. As reported by the study authors, MNCL has a significant increasing trend over time and was the most common cause of unscheduled deaths and/or morbidity. In many of the treated rats, MNCL was detected at a very early stage and was limited to an increase in the mononuclear cells in the hepatic sinusoids.

Table 3-5. Incidence of MNCL in F344 Rats Exposed to DINP for 2 Years (Lington et al., 1997; Bio/dynamics, 1986)

Lesion	Dose Group (mg/kg-day) (ppm)			
	Control	15 Male / 18 Female (300)	152 Male / 184 Female (3,000)	307 Male / 375 Female (6,000)
Males[a]	33/81 (41%)	28/80 (35%)	48/80* (60%)	51/80* (64%)
Females[a]	22/81 (27%)	20/81 (25%)	30/80* (38%)	43/80* (54%)

Source: Table 8 in Lington et al. (1997)
[a] Number of animals with lesion/ total number of animals examined. Percent lesion incidence in parentheses.
* Statistically significant at $p < 0.05$ when compared to the control incidence using Fisher's Exact test; statistical analysis performed by Lington et al. (1997).

In a study by Covance Labs (1998b), the incidences of MNCL in male and female rats receiving the 6,000 and 12,000 ppm concentrations of DINP in the diet were significantly increased with statistically significant dose-related trends (Table 3-6). The incidences of MNCL in the recovery groups were also significantly greater than in the controls. There is some evidence that the onset of MNCL was earlier in treated males. MNCL was first detected in the 6,000 ppm group via an unscheduled death at study day 352. In comparison, MNCL was first detected in the control group at an interim sacrifice at day 549. Decreases in hemoglobin concentration and red blood cell numbers and a statistically significant increase in mean spleen weight in both male and female rats were correlated with the incidence of MNCL. Between 31 and 60 percent of unscheduled deaths in the study were attributable to MNCL (Table 3-7), demonstrating that this lesion is life-threatening in rats treated with DINP.

A Histopathology Peer Review and a Pathology Working Group (PWG) review (EPL, 1999) was conducted on selected lesions of the liver and spleen observed in F344 rats in the 2-year bioassays reported by Lington et al. (1997) and Covance Labs (1998b). The PWG review evaluated the significance of spongiosis hepatis, foci of cellular alteration, primary hepatocellular neoplasms in the liver, and the significance of MNCL. Notably, the results of the Histopathology Peer Review and PWG (EPL, 1999) generally confirmed the original findings of the study pathologist(s), including incidence of MNCL in F344 rats in both studies. PWG findings are further discussed in Appendix A.

Table 3-6. Incidence of MNCL in F344 Rats Exposed to DINP in the Diet for 2 Years (Covance Labs, 1998b)[a b c]

Sex	Dose Group mg/kg-day (ppm)					
	Control	29 M / 36 F (500)	88 M / 109 F (1,500)	359 M / 442 F (6,000)	733 M / 885 F (12,000)	High-Dose / Recovery[b] 637 M/ 774 F (12,000)
Males	22/65 (34%)	23/50 (46%)	21/50 (42%)	32/65* (49%)	30/65* (46%)	31/50*[d] (62%)
Females	17/65 (26%)	16/49 (33%)	9/50 (18%)	30/65* (46%)	29/65* (45%)	24/50*[d] (48%)

Source: U.S. CPSC (2001) text pages 68–71 and Appendix B.
M = male; F = female
* = statistically significant at $p < 0.05$ by one or more of the following: Fisher's Exact test, Poly-3, Logistic Regression, or Life Table analysis.
[a] Analysis of individual animal data as performed by the National Toxicology Program and reported in the text and Appendix B of U.S CPSC (2001).
[b] The high-dose/recovery group received 12,000 ppm for 78 weeks, followed by a 26-week recovery period during which the animals received basal diet alone.
[c] Number of animals with neoplasm/ total number of animals examined. Percent tumor incidence in parentheses. Based on extraction and analysis of individual animal data as reported in U.S. CPSC (2001). Overall incidence for control, 6,000 ppm and 12,000 ppm groups (n = 65) includes incidence data for unscheduled deaths, interim sacrifice at week 78, and terminal sacrifice. Overall incidence for the remaining groups includes incidence data for unscheduled deaths and terminal sacrifice.
[d] Statistical significant at $p < 0.05$ by Fisher's Exact test conducted by Syracuse Research Corporation.

Table 3-7. MNCL as a Cause of Unscheduled Death in F344 Rats Exposed to DINP in the Diet (Covance Labs, 1998b)

Sex	Dose Group mg/kg-day (ppm)					
	Control	29 M / 36 F (500)	88 M / 109 F (1,500)	359 M / 442 F (6,000)	733 M / 885 F (12,000)	Recovery[a] 637 M / 774 F (12,000)
Males	7/22[b] (32%)	8/23 (35%)	7/21 (33%)	16/32 (50%)	18/30 (60%)	14/31 (45%)
Females	7/17 (41%)	5/16 (31%)	3/9 (33%)	12/29 (41%)	13/30 (43%)	12/24 (50%)

Source: Compiled from incidence data and death comments in Table 10E (pages 365 and 381) in Covance Labs (1998b).
M = male; F = female
[a] The high-dose/recovery group received 12,000 ppm for 78 weeks, followed by a 26-week recovery period during which test animals received basal diet alone.
[b] Number of deaths attributed to MNCL/total number of deaths; percentage of deaths attributable to MNCL in parentheses.

3.2.2.1 Conclusions on Mononuclear Cell Leukemia

The incidence of MNCL was significantly elevated in male and female F344 rats exposed to DINP in the diet when compared to study control animals in two independent carcinogenicity studies (Covance Labs, 1998b; Lington et al., 1997). In Lington et al. (1997), incidences of MNCL were statistically significantly increased at 152 and 307 mg/kg-day in the males (60 to 64 percent in treated rats versus 41 percent in concurrent controls) as well as in the females at 184 and 375 mg/kg-day (38 to 54 percent in treated rats versus 27 percent in concurrent controls). In the 2-year study in F344 rats conducted by Covance Labs (1998b), incidences of MNCL were significantly increased at 359 and 733 mg/kg-day in the treated males (46 to 62 percent incidence) compared to concurrent controls (34 percent incidence) as well as in the treated females at 442 and 885 mg/kg-day (45 to 48 percent) compared to concurrent controls (26 percent incidence). Inconsistent with findings from the two chronic studies of F344 rats, MNCL was not observed in male or female SD rats treated with up to 553 to 672 mg/kg-day DINP for 2

years (Bio/dynamics, 1987) or male and female B6C3F1 mice treated with up to 1,560 to 1,888 mg/kg-day DINP for two years (Covance Labs, 1998a).

MNCL is a spontaneously occurring neoplasm of the hematopoietic system that reduces lifespan and is one of the most common tumor types occuring at a high background rate in the F344 strain of rat (Thomas et al., 2007). Historical control data from NTP have demonstrated an increase in the spontaneous background incidence of MNCL in untreated male and female F344 rats from 7.9 and 2.1 percent in males and females, respectively, in 1971 to 52.5 and 24.2 percent in males and females, respectively, from 1995 through 1998 (Thomas et al., 2007). Spontaneous incidence of MNCL in other strains of rat appear to be rare. Brix et al. (2005) report the incidence of MNCL in female Harlan SD rats to be 0.5 percent in NTP 2-year studies. Further, MNCL does not appear to occur naturally in mice (Thomas et al., 2007).

Given the high and variable background rate of MNCL in F344 rats, it is important to consider concurrent control data, historical control data, and time to onset of MNCL to assist in determining whether observed increases in MNCL are treatment-related.

EPA acknowledges that MNCL has a high background incidence in F344 rats as is noted by concurrent control incidence of 26 to 41 percent in the two studies described above (Covance Labs, 1998b; Lington et al., 1997). The incidence of MNCL was significantly elevated in male and female rats exposed to DINP in the diet when compared to concurrent controls in these studies; however, no historical control data from the performing laboratories were provided. EPA's *Guidelines for Carcinogen Risk Assessment* (2005) state that the most relevant historical control data comes from the same laboratory and supplier and are within 2 to 3 years of the study under review, and that other historical control data should be used with extreme caution. Lack of relevant laboratory historical control data for incidence and time to onset of MNCL make it challenging to determine if the increase in MNCL observed in high-dose F344 rats treated with DINP, which was statistically significant compared to concurrent controls, is treatment-related and is a source of uncertainty.

The limited information available indicates that time to onset of MNCL was shorter in DINP-treated animals compared to concurrent controls. In Lington et al. (1997), the study authors reported that MNCL has a significant increasing trend over time and was the most common cause of unscheduled deaths and/or morbidity. In many of the treated rats, MNCL was detected at a very early stage but was limited to an increase in the mononuclear cells in the hepatic sinusoids. Similar to the Lington study, in the 2-year study in rats conducted by Covance Labs (1998b), there is some evidence that the onset of MNCL was earlier in treated males, with the first detected in the 359 mg/kg-day group via an unscheduled death at study day 352 compared to the first detected in the control group at an interim sacrifice at day 549.

Another source of uncertainty is lack of MOA information for induction of MNCL in F344 rats. The MOA for induction of MNCL in F344 rats is unknown. Lack of MOA information makes it difficult to determine human relevancy. There is additional uncertainty related to the human correlate to MNCL in F344 rats. Some researchers have suggested that based on the biological and functional features in the F344 rat, MNCL is analogous to large granular lymphocyte (LGL) in humans (Caldwell et al., 1999; Caldwell, 1999; Reynolds and Foon, 1984). There are two major human LGL leukemias, including CD3+ LGL leukemia and CD3− LGL leukemia with natural killer cell activity (reviewed in (Maronpot et al., 2016; Thomas et al., 2007). Thomas et al. (2007) contend that MNCL in F344 rats shares some characteristics in common with aggressive natural killer cell leukemia (ANKCL) in humans, and that ANKCL may be a human correlate. However, Maronpot (2016) point out that ANKCL is extremely rare with less than 98 cases reported worldwide, and its etiology is related to infection with Epstein-Barr

virus, not chemical exposure. This is in contrast to MNCL in F344 rats, which is a more common form of leukemia and is not associated with a viral etiology. However, under EPA's *Guidelines for Carcinogen Risk Assessment* (U.S. EPA, 2005), site concordance is not always assumed between animals and humans.

EPA considers the available data inadequate for delineation of a plausible sequence of events leading to development of MNCL in rats exposed to DINP. Therefore, the significance of MNCL and its biological relevance for human cancer risk remains uncertain. Other regulatory agencies have also considered the human relevance of MNCL. Generally, other agencies such as Australia NICNAS (2012)[1] Health Canada (EC/HC, 2015),[2] U.S. CPSC (2010),[3] and ECHA (2013)[4] have concluded that MNCL observed in F344 rats is not human relevant or has unclear human relevance and refrained from using MNCL to predict cancer risk in humans. In contrast, California OEHHA (Tomar et al., 2013) lists MNCL in F344 rats as one of the tumor types to support the Proposition 65 listing of DINP; however, OEHHA does not appear to draw any specific conclusions related to the MOA underlying MNCL or its human relevance.

Overall, considerable scientific uncertainty remains. Therefore, EPA did not consider it appropriate to derive quantitative estimates of cancer hazard for data on MNCL from these two studies in F344 rats.

3.2.3 Kidney Tumors

Statistically significant increased incidence of kidney tumors have been observed in one 2-year dietary study of F344 rats (Covance Labs, 1998b). Malignant renal tubule cell carcinomas were detected in two high-dose (733 mg/kg-day) male rats and four males treated with 637 mg/kg-day DINP for 78 weeks followed by a 26-week recovery period (Table 3-8). However, incidence of renal tubular carcinomas only reached statistical significance in the recovery group.

[1] Australia NICNAS concluded "In rat carcinogenicity studies, increased incidences of MCL, kidney and liver neoplasia were observed. MCL was observed in DINP toxicological studies with Fischer 344 rats but not with Sprague Dawley rats. MCL is a common neoplasm in Fischer 344 rats with no comparable tumour type in humans and its increased incidence after chronic exposure to some substances is a strain-specific effect (Caldwell, 1999). Therefore, MCL observed in Fischer 344 rats is not regarded as relevant to humans" (p. 49 of (NICNAS, 2012)).

[2] Health Canada concluded "Mononuclear cell leukemia of the spleen was also reported in Fischer rats. However, this type of lesion is likely specific to aging rats of this strain and is unlikely to be relevant to humans (Health Canada 2015d)." (p. 95 of (Health Canada, 2015)).

[3] U.S. CPSC concluded "Elevated incidence of MNCL is a common finding in chronic studies in Fischer rats. Due to its high background rate, MNCL is often considered to be of uncertain relevance in the evaluation of the cancer hazard in humans. Furthermore, no hematopoietic neoplasms were found in Sprague-Dawley CD rats treated with DINP-A (Bio/dynamics, 1986) or in mice treated with DINP-1 (Caldwell, 1999). Therefore, MNCL will not be used to predict cancer risk in humans" (p. 82 of (U.S. CPSC, 2010)).

[4] ECHA concluded "With regard to MNCL, the review by (Thomas et al., 2007) suggests that unlike previously thought there might be a human counterpart to MNCL in rats. The probability that the MNCL seen in the Exxon and Aristech studies would be a result of chance findings seems low. Nevertheless, the increased incidences of MNCL remain difficult to interpret in the light of the high and variable background incidences and the unclear relevance to humans. DINP is not genotoxic, and it is argued (Caldwell, 1999) that MNCL follows a threshold mode of action. The available information does not allow to draw definite conclusions on the matter. However, as a reasonable approach it would be possible to conclude that the MNCL findings further strengthen the selected NOAELs for repeated dose toxicity (15 and 88 mg/kg bw/day). Since such conclusion would not influence the outcome of the current risk assessment, the endpoint is not taken further to the risk characterization step" (p. 98 of (ECHA, 2013)).

Table 3-8. Incidence of Kidney Tumors in Male F344 Rats Exposed to DINP in the Diet for 2 Years (Covance Labs, 1998b)[a b c]

Lesion	Dose Group mg/kg-day (ppm)					
	Control	29 M / 36 F (500)	88 M / 109 F (1,500)	359 M / 442 F (6,000)	733 M / 885 F (12,000)	High-Dose/ Recovery 637 M / 774 F (12,000)
Renal tubular carcinoma	0/65 (0%)	0/55 (0%)	0/55 (0%)	0/65 (0%)	2/65 (3.1%)	4/50* (8.0%)

Source: U.S. CPSC (2001) text pages 68–71 and Appendix B.
* = statistically significant at p<0.05 by one or more of the following: Fisher's Exact test, Poly-3, LogisticRegression, or Life Table analysis.
[a] Analysis of individual animal data as performed by the National Toxicology Program and reported in the textand Appendix B of U.S. CPSC (2001)
[b] The high-dose/recovery group received 12,000 ppm for 78 weeks, followed by a 26-week recovery periodduring which they received basal diet alone.
[c] Number of animals with neoplasm/ total number of animals examined. Percent tumor incidence in parentheses. Based on extraction and analysis of individual animal data as reported in U.S. CPSC (2001)
Overall incidence for control, 6,000 ppm and 12,000 ppm groups (n = 65) includes incidence data for unscheduled deaths, interim sacrifice at week 78 and terminal sacrifice. Overall incidence for the remaining groups includes incidence data for unscheduled deaths and terminal sacrifice.

Lington et al. (1997) reported the incidence data for selected transitional cell carcinomas, transitional cell adenomas, and tubular cell carcinomas and adenomas in the kidney (Table 3-9). Renal tubular cell carcinomas were observed in one male in the low-dose group and two males in the high-dose group and renal transitional cell carcinoma was observed in three male rats in the mid-dose group. However, neither tumor type was statistically significantly increased. Further, no preneoplastic renal lesions were detected in rats of either sex and no neoplastic lesions were detected in the kidneys of female rats.

Kidney tumors have not been observed in male or female SD rats treated with up to 553 to 672 mg/kg-day DINP for 2 years (Bio/dynamics, 1987) or male and female B6C3F1 mice treated with up to 1,560 to 1,888 mg/kg-day DINP for 2 years (Covance Labs, 1998a).

Table 3-9. Incidence of Kidney Tumors in F344 Rats Exposed to DINP for 2 Years (Lington et al., 1997; Bio/dynamics, 1986)

Lesion	Dose Group mg/kg-day (ppm)			
	Control	15 M / 18 F (300)	152 M / 184 F (3,000)	307 M / 375 F (6,000)
Males[a]				
Transitional cell carcinoma	0/81 (0%)	0/80 (0%)	3/80 (3.8%)	0/80 (0%)
Transitional cell adenoma	0/81 (0%)	0/80 (0%)	0/80 (0%)	0/80 (0%)
Tubular cell carcinoma	0/81 (0%)	1/80 (1.3%)	0/80 (0%)	2/80 (2.5%)
Tubular cell adenoma	0/81 (0%)	0/80 (0%)	0/80 (0%)	0/80 (0%)
Females[a]				
Transitional cell carcinoma	0/81 (0%)	0/81 (0%)	0/80 (0%)	0/80 (0%)
Transitional cell adenoma	0/81 (0%)	0/81 (0%)	0/80 (0%)	0/80 (0%)
Tubular cell carcinoma	0/81 (0%)	0/81 (0%)	0/80 (0%)	0/80 (0%)
Tubular cell adenoma	0/81 (0%)	0/81 (0%)	0/80 (0%)	0/80 (0%)

Source: Table 8 in Lington et al. (1997)
M = male; F = female
[a] Number of animals with lesion/ total number of animals examined. Percent lesion incidence in parentheses.
[b] Statistically significant at $p < 0.05$ when compared to the control incidence using Fisher's Exact test;statistical analysis performed by Lington et al. (1997).

3.2.3.1 Conclusions on Kidney Tumors

Two tumor types have been reported in the kidneys of male F344 rats following chronic oral exposure to DINP, including renal transitional cell carcinomas and renal tubule cell carcinomas.

Renal transitional cell carcinoma, an uncommon tumor type in rats, has been reported in two out of four rodent carcinogenicity studies. Lington et al. (1997) report transitional cell carcinoma in 3/80 mid-dose (151 mg/kg-day) male F344 rats. However, the response was not statistically significant and did not occur in a dose-dependent manner (not observed in high-dose males [307 mg/kg-day]). Similarly, in a study conducted by Covance Labs (1998b), transitional cell carcinoma was detected in 1/65 male F344 rats treated with 359 mg/kg-day DINP; however, the response was not statistically significant and did not occur in high-dose (733 mg/kg-day) or high-dose recovery (637 mg/kg-day) males. Renal transitional cell carcinoma was not reported in male SD rats treated with up to 553 mg/kg-day DINP (Bio/dynamics, 1987) or male B6C3F1 mice treated with up to 1,560 mg/kg-day DINP (Covance Labs, 1998a), and has not been reported in female mice or rats at any dose. Given the lack of dose-response and statistical significance across available studies, the low incidence of renal transitional cell carcinomas observed in male F344 rats is considered to be of uncertain toxicological significance.

Renal tubule cell carcinomas have also been reported in two of four rodent carcinogenicity studies. In the study conducted in F344 rats by Covance Labs (1998b), renal tubule cell carcinoma was observed in 2/65 high-dose (733 mg/kg-day) males and 4/50 recovery high-dose (637 mg/kg-day) males compared to 0/65 in the control group. The response in recovery males was statistically significant relative to the control group. In the Lington et al. (1997) study, a non-statistically significant increase in renal tubule cell carcinoma was observed in 1/80 low-dose (15 mg/kg-day), 0/80 mid-dose (152 mg/kg-day), and

2/80 high-dose (307 mg/kg-day) male F344 rats. Renal tubule cell carcinomas were not observed in SD rats treated with up to 533 mg/kg-day DINP (Bio/dynamics, 1987) or in male B6C3F1 mice treated with up to 1,560 mg/kg-day DINP (Covance Labs (1998a)). No preneoplastic or neoplastic lesions were observed in female rats or mice at any dose.

The male rat specific alpha 2u-globulin (α_{2u}-globulin) MOA has been implicated as being causative of renal tubule cell carcinomas. U.S. EPA (1991) [5] and IARC (1995) [6] have published related criteria for establishing an α_{2u}-globulin MOA for this tumor type. EPA does not consider kidney tumors arising through a α_{2u}-globulin MOA to be human relevant (U.S. EPA, 1991). Data are available to support many, but not all of, the EPA and IARC criteria for an α_{2u}-globulin MOA. The three specific criteria for establishing an α_{2u}-globulin MOA include demonstration (1) that renal tubule cell carcinomas only occur in male rats, (2) immunohistochemical evidence, and (3) histological evidence. In the case of DINP, these three requisites have been met across four chronic studies: kidney tumors were only observed in male rats, and the weight of evidence indicates that DINP is not genotoxic. Much of the additional evidence supporting a α_{2u}-globulin MOA comes from Caldwell et al.'s (1999) retrospective evaluation of archived kidney tissue taken from the 12-month interim sacrifice from the chronic rat study conducted by Lington et al. (1997). Caldwell et al. report a dose-dependent increase in the accumulation of α_{2u}-globulin and increased droplet size in the kidneys of high-dose male (but not female) rats. Cell proliferation measured via immunohistochemical staining for proliferating cell nuclear antigen in kidney sections was not statistically significantly elevated in high-dose males (125 percent of controls) or females (112 percent of control).

Photomicrographs for proliferating cell nuclear antigen and α_{2u}-globulin staining showed foci of proliferating cells and α_{2u}-globulin accumulating in proximal tubule cells of the P2 segment; however, some cell proliferation was also observed in P1 and P3 cells. Histopathologic re-analysis of kidney sections showed a dose-dependent increase in minimal tubular regeneration (incidence 6/9, 10/10, 9/10, and 10/10 in control, low-, mid-, and high-dose males, respectively) and minimal tubular epithelial hypertrophy (0/9, 0/10, 10/10, and 9/10 in control, low-, mid-, and high-dose males, respectively). Tubular epithelial hypertrophy was not observed in control or high-dose females; however, minimal tubular regeneration was observed in 1/10 high-dose female. Collectively, Caldwell et al. concluded that findings were consistent with an α_{2u}-globulin MOA.

Additional histopathological findings consistent with an α_{2u}-globulin MOA have been noted. For example, a dose-related increase in incidence of mineralization of renal papilla was reported in the kidneys of male, but not female, F344 rats in the chronic study conducted by Covance Labs (1998a).

Generally, EPA's three primary criteria for establishing an α_{2u}-globulin MOA have been met. However, data are not available to inform all of the IARC criteria and several findings raise uncertainty. First, reversible binding of DINP to α_{2u}-globulin has not been demonstrated. Additionally, chronic exposure to

[5] EPA criteria include (1) an increase in number and size of hyaline (protein) droplets in kidney proximal tubule cells of treated male rats; (2) immunohistochemical evidence of α_{2u}-globulin accumulating protein in the hyaline droplets; and (3) histopathological evidence of kidney lesions associated with α_{2u}-globulin nephropathology. The Agency also acknowledges additional information that may be useful for the analysis that are consistent with IARC criteria (*e.g.*, chemical is negative for genotoxicity, reversible binding of chemical to α_{2u}-globulin, sustained cell division in the proximal tubule of the male rat).

[6] IARC criteria include (1) tumors occur only in male rats, (2) acute exposure exacerbates hyaline droplet formation, (3) α_{2u}-globulin accumulates in hyaline droplets, (4) intermediate lesions include granular casts and linear papillary mineralization, (5) absence of hyaline droplets and other histopathological changes in female rats and mice, and (6) negative for genotoxicity. Additional supporting evidence includes (1) reversible binding of chemical to α_{2u}-globulin, (2) increased sustained cell proliferation in proximal tubule (P2 segment), and (3) dose-response relationship between hyaline droplet severity and renal tumor incidence.

DINP has been shown to increase absolute and relative kidney weight in both male and female rats (Covance Labs, 1998b; Lington et al., 1997; Bio/dynamics, 1987) as well as cause a significant dose-related increase in chronic progressive nephropathy in female mice (Covance Labs, 1998a); however, this lesion was not elevated in the high-dose recovery group females, indicating its reversibility. These kidney effects cannot be explained by an α_{2u}-globulin MOA.

Other agencies have evaluated the renal tubule cell carcinoma MOA. The U.S. CPSC (2010),[7] Australia NICNAS (2012),[8] and ECHA (2013) [9] have all concluded that the renal tubule cell carcinomas observed in male rats occur through an α_{2u}-globulin MOA that is not relevant for use in human health risk assessment. Although Health Canada (EC/HC, 2015) [10] concluded that certain effects observed in the kidneys of female rats and mice cannot be explained by an α_{2u}-globulin MOA, Health Canada considered the kidney tumors in rodents to be of little or unclear relevance to humans. In contrast, California OEHHA concluded that "α_{2u}-globulin accumulation in the renal tubules of male rats do not explain the renal tubule carcinomas observed in DINP-exposed rats" and that renal tubule cell carcinomas were one of the tumor types listed to support the Proposition 65 listing of DINP (Tomar et al., 2013).

Although some uncertainty remains, much of the available literature supports an α_{2u}-globulin MOA to explain the incidences of renal tubule cell carcinomas observed in male rats exposed to DINP. EPA does not consider kidney tumors arising through a α_{2u}-globulin MOA to be human relevant (U.S. EPA, 1991). Therefore, EPA did not consider it appropriate to derive quantitative estimates of cancer hazard for data on kidney tumors observed in these studies.

3.2.4 Other Tumors

The carcinogenicity of DINP was investigated in a Good Laboratory Practice (GLP)-compliant 2-year dietary study in SD rats by Bio/dynamics (1987). Incidence data for select histopathological observations and results from statistical analyses are provided in Table 3-10. In addition to findings in the liver and kidney previously discussed, tumors were noted in the pancreas, testes, and uterus. However, for these organs histopathologic examination was only conducted on control and high-dose rats.

Pancreatic islet cell adenomas (8/70 treated vs 6/70 controls) and carcinomas (4/70 treated vs 1/70 controls) were observed at a slightly higher incidence in the high-dose males compared to controls, and the nonsignificant incidences of pancreatic tumors were considered to be within the range of normal

[7] The U.S. CPSC concluded "A small number of renal tubular cell carcinomas were observed only in males exposed to 1.2 percent DINP. Furthermore, there is experimental evidence that these tumors arose by a mechanism involving the accumulation of α2u-globulin (Caldwell et al. 1999). α2u-Globulin is a protein that is specific to the male rat. Renal tubular cell tumors induced by this mechanism are not considered relevant to human risk assessment (Schaeffer 1991)" (p.81 of (U.S. CPSC, 2010))

[8] Australia NICNAS concluded "kidney tumours in male rats appear consistent with a specific gender- and species-specific alpha 2μ-globulin accumulation mechanism that is not regarded as relevant to humans" (p. 49 of (NICNAS, 2012)).

[9] ECHA concluded "The available new information on the carcinogenicity of DINP further supports the conclusions of the EU Risk Assessment concerning renal tumors (EC 2003a). These neoplasms are assumed to have modes of actions which are not considered to be relevant for humans (alpha-2u-globulin)" (p. 98 of (ECHA, 2013)).

[10] Health Canada concluded "Renal tubular cell carcinomas were also reported in one chronic study in rats. It has been suggested that the mechanism responsible for these tumours was related to accumulation of α2u-globulin, a protein specific to the male rat (Health Canada 2015d). While this type of neoplastic lesion has not been observed in female rats, increased kidney weights accompanied by histopathological changes were noted in female rats exposed for 2 years (Covance Labs, 1998b) and treatment-related nephropathy was noted in female mice in another chronic study conducted by the same author (Covance Labs, 1998a). Those kidney effects cannot be explained by an α2u-globulin mode of action. Overall, findings in the kidneys of rodents could be considered of little or unclear relevance to humans" (p. 95 of (EC/HC, 2015)).

biological variation. Furthermore, in the females, pancreatic islet cell adenomas were only observed in one high-dose and one control animals, and no pancreatic islet cell carcinomas were noted in females.

In the testes, incidences of interstitial cell hyperplasia were significantly increased at the high-dose (22/70) compared to controls (4/70) and were also reported to exceed historical controls. Testicular interstitial cell tumors was increased at the high-dose (7/70) compared to controls (2/70); however, the increase in tumors was not statistically significant and was reported to be within the range of historical controls.

Similarly, in the uterus, incidence of endometrial hyperplasia was significantly increased at the high-dose (13/69) compared to controls (2/70). Endometrial adenocarcinoma was observed in 2/69 females at the high-dose compared to 0/70 controls; however, the increase in tumors was not statistically significant.

It is plausible that the significantly increased incidences of hyperplasia noted in the testes and uterus at the high-dose are proliferative responses that can lead to the slight (not significant) increases in testicular and uterine tumors. However, the fact that the incidences of these tumors is low and, for the testes data, within the range of historical controls, there is not strong evidence of a carcinogenic response. Furthermore, the lack of examination of the low- and mid-dose groups limits the examination of dose-dependency for the cancer incidence in these organs and may miss low-dose effects on any hormonally-influenced tumors or receptor-mediated carcinogenicity. Finally, tumors in the testes and uterus were not noted in other chronic studies of DINP in rodents. Overall, there is too much uncertainty for EPA to consider using these data to derive quantitative estimates of cancer risk.

Table 3-10. Incidence of Tumors in Pancreas, Testes, and Uterus in SD Rats Exposed to DINP for 2 Years (Bio/dynamics, 1987)[a]

Observation		Dose Group mg/kg-day (ppm)							
		Males				Females			
		0	27 (500)	271 (5,000)	553 (10,000)	0	33 (500)	331 (5,000)	672 (10,000)
Pancreas									
No. examined		70	0	0	70	69	0	0	70
Pancreatic islet cell adenoma	–	6	–	–	8	1	–	–	1
Pancreatic islet cell carcinoma	–	1	–	–	4	0	–	–	0
Testes									
No. examined		69	0	0	70	N/A	N/A	N/A	N/A
Interstitial cell hyperplasia	Total	4	–	–	22*	–	–	–	–
	Unilateral	3	–	–	9	–	–	–	–
	Bilateral	1	–	–	13	–	–	–	–
Interstitial cell tumors	Total	2	–	–	7	–	–	–	–
	Unilateral	2	–	–	6	–	–	–	–
	Bilateral	0	–	–	1	–	–	–	–
Uterus									
No. examined		N/A	N/A	N/A	N/A	70	0	0	69
Endometrial hyperplasia	–	–	–	–	–	2	–	–	13*
Endometrial adenocarcinoma	–	–	–	–	–	0	–	–	2

* $p < 0.05$ based on a two-tailed Fisher's exact test calculated for this review.
[a] Data in this table indicate all animals assessed for histopathology throughout the study; that is, including the interim sacrifice, the terminal sacrifice, and unscheduled deaths. For late-developing tumors (pancreatic islet cell tumors, testicular interstitial cell tumors), statistical analysis was performed excluding animals that died or were sacrificed up to 12 months, leaving n = 57, 57, 59, 59 in males and n = 59, 56, 60, 59 in females in the control, low-, mid- and high-dose groups, respectively. Data from Appendix K of (Bio/dynamics, 1987).

4 POSTULATED MODE OF ACTION FOR LIVER TUMORS IN RATS AND MICE

As described in Section 3.2.1, available studies provide consistent evidence that chronic oral exposure to DINP can cause treatment-related hepatocellular adenomas and/or carcinomas in male and female F344 and SD rats and male and female B6C3F1 mice. EPA further considers the weight of evidence for liver carcinogenesis and its underlying MOA in Sections 4.1 through 4.9.

4.1 Postulated Mode of Action in Rats and Mice

Studies have demonstrated that DINP can activate peroxisome proliferator-activated receptor alpha (PPARα) in hepatocytes and cause hepatocellular adenomas and carcinomas in mice and rats. Existing assessments of DINP by U.S. CPSC (2014, 2010), Health Canada (ECCC/HC, 2020; EC/HC, 2015; Health Canada, 2015), ECHA (2013), and NICNAS (2012) have postulated that DINP causes liver tumors in rats and mice through a PPARα MOA. PPARα is a nuclear receptor that controls transcription of genes involved in fatty acid β-oxidation and peroxisome proliferation. PPARα activation in hepatocytes in rodent models can cause hepatocellular cancer through a non-genotoxic MOA that involves activation of Kupfer cells. Activated Kupfer cells secrete cytokines such as TNFα, IL-1α, and IL-1β that influence hepatocyte growth and fate. As discussed by Corton et al. (2018; 2014), studies have demonstrated that Kupffer cell activation following PPARα activation plays a crucial role in several tumor precursor effects. These effects include increased DNA synthesis and cell proliferation in both normal and preneoplastic hepatocytes, as well as suppression of apoptosis. Altered cell growth and survival can facilitate clonal expansion of initiated cells leading to the selective clonal expansion of preneoplastic foci cells and ultimately tumor formation.

The PPARα MOA for liver tumorigenesis considered by EPA is described further by Corton et al. (2018; 2014). Consistent with U.S. *EPA Guidelines for Carcinogen Risk Assessment* (U.S. EPA, 2005) and the IPCS *Mode of Action Framework* (IPCS, 2007), EPA further evaluated the postulated PPARα MOA for liver tumors, as well as evidence for other plausible MOAs for DINP.

The PPARα MOA includes the following sequence of key events (KEs):

- KE1: activation of PPARα in hepatocytes;
- KE2: alterations in cell growth pathways (*e.g.,* Kupfer cell activation leading to increased cytokine (*e.g.,* TNFα, IL-1α, IL-1β) secretion;
- KE3: perturbation of cell growth and survival (*i.e.,* increased cell proliferation and inhibition of apoptosis); and
- KE4: selective clonal expansion of preneoplastic foci cells leading to the apical outcome, hepatocellular adenomas, and carcinomas.

Several modulating factors associated with the PPARα MOA have also been proposed, including increases in reactive oxygen species (ROS) and activation of nuclear factor kappa B (NF-κB) (Corton et al., 2018). These modulating factors are not considered necessary to induce liver tumorigenesis but may modulate the dose-response behavior or the probability of inducing one or more KEs (Corton et al., 2014).

Evidence for each KE (Sections 4.1.1 to 4.1.4) and EPA's analyses of dose-response (Section 4.1.5); temporality (Section 4.3); strength, consistency, and specificity (Section 4.4); biological plausibility and coherence (Section 4.5); other carcinogenic MOAs (Section 4.6); uncertainties and limitations (Section 4.7); weight of scientific evidence for liver tumors (Section 4.8) are presented below.

4.1.1 Key Event 1: PPARα Activation

PPARα activation can be assessed using trans-activation assays or by measuring specific events associated with PPARα activation, such as increased expression of genes involved in beta oxidation or peroxisome proliferation, increased activity of palmitoyl-CoA oxidase, increased peroxisomal beta oxidation (PBOX), and/or peroxisome proliferation in hepatocytes. Activation of PPARα in hepatic cells by DINP has been consistently demonstrated in five *in vivo* studies of mice and four *in vivo* studies of rats. No evidence of PPARα activation in hepatic cells was observed in two *in vivo* studies of monkeys. Additionally, four *in vitro* studies investigating PPARα activation are available. Available data for KE1 are discussed further below.

Evidence from In Vitro Studies

Four *in vitro* studies of DINP are available that consistently demonstrate that rat and mouse hepatocytes are more sensitive to PPARα activation compared to human and monkey hepatocytes. Bendford et al. (1986) demonstrated that *in vitro* treatment of primary rat hepatocytes isolated from adult Wistar rats with concentrations of MINP ranging from 0.1 to 0.5 mM for 3 days caused large (up to approximately 750 percent) dose-dependent increases in palmitoyl-CoA oxidation and laurate hydroxylation activity. Comparatively, smaller (approximately 200 to 300 percent) increases in palmitoyl-CoA oxidation and laurate hydroxylation activity were observed in primary marmoset hepatocytes under similar experimental conditions. Hasmall et al. (1999) demonstrated that treatment of primary rat hepatocytes isolated from male F344 rats with 250 and 500 µM (but not 750 µM) DINP can induce increases in PBOX activity. In contrast, no increase in PBOX was noted in primary human hepatocytes treated with up to 750 µM DINP under similar experimental conditions. Similarly, Shaw et al. (2002) report dose-related induction of PBOX activity in primary rat hepatocytes isolated from male F344 rats treated with 150 to 250 µM MINP, however, PBOX activity was not increased in primary human hepatocytes treated with up to 250 µM MINP under similar experimental conditions. Finally, Bility et al. (2004) demonstrated that mouse PPARα is more inducible and activated at lower concentrations compared to human PPARα in mouse 3T3-L1 fibroblasts transfected with a plasmid encoding mouse or human PPARα luciferase reporter (lowest activation concentration: 3 and 10 µM for mouse and human, respectively; maximal fold-induction: 27.1 and 5.8 for mouse and human, respectively).

Evidence from In Vivo Studies of Rats

Three studies of rats provide consistent evidence of treatment-related increases in PPARα activation following oral exposure to DINP. Smith et al. (2000) reported treatment-related increases in hepatic PBOX in male F344 rats fed diets containing up to 12,000 ppm DINP (approximately 1,200 mg/kg-day) for 2 or 4 weeks; however, no change was observed in the low-dose group (approximately 100 mg/kg-day). Similarly, BIBRA (1986) reported increased hepatic cyanide-insensitive palmitoyl-CoA oxidation levels and hepatic lauric acid 11- and 12-hydroxylase activities in male and female F344 rats treated with high-doses of DINP for 21-days (biomarkers of PPARα activation increased in males and females starting at 639 and 1,198 mg/kg-day, respectively). Finally, cyanide-insensitive palmitoyl-CoA oxidase activity was increased in the livers of male and female F344 rats treated with 733 (males) to 885 (females) mg/kg-day DINP after 1, 2, 13, and 104 weeks of exposure to DINP, as well as for females treated with 442 mg/kg-day DINP for 104 weeks (Covance Labs, 1998b). In contrast, no evidence of peroxisome proliferation (evaluated via electron microscopy) was reported in hepatocytes from male or female F344 rats treated with up to 307 (males) to 375 (females) mg/kg-day DINP for 2 years (Lington et al., 1997).

Evidence from In Vivo Studies of Mice

Five studies of mice provide consistent evidence of treatment-related increases in PPARα activation following oral exposure to DINP. Smith et al. (2000) reported treatment-related increases in hepatic

PBOX in male B6C3F1 mice fed diets containing up to 6,000 ppm DINP (approximately 900 mg/kg-day) for 2 or 4 weeks; however, no change was observed in the low-dose group at either timepoint (approximately 75 mg/kg-day). In a second study, Kaufmann et al. (2002) reported dose-related increases in the number and volume of peroxisomes and hepatic cyanide-insensitive palmitoyl-CoA oxidation activity in male B6C3F1 mice after 4 weeks at doses as low as 117 mg/kg-day, while similar changes were observed in female mice starting at 546 mg/kg-day DINP. Similarly, Valles et al. (2003) reported treatment related increases in hepatic palmitoyl-CoA oxidase activity in male and female B6C3F1 mice treated with diets containing 4,000 to 8,000 ppm DINP (approximately 600 to 1,200 mg/kg-day) for 2 weeks. In a study by Hazleton Labs (1992), large (albeit not always statistically significant), dose-related, increases in hepatic cyanide-insensitive palmitoyl CoA oxidation were observed in male and female B6C3F1 mice treated with 365 and 2,600 mg/kg-day DINP for 4, 31, and 91 days. Similarly, large increases in hepatic cyanide-insensitive palmitoyl-CoA oxidation activity were observed in male and female B6C3F1 mice treated with 1,560 (males) to 1,888 (females) mg/kg-day DINP for 79 and 105 weeks (Covance Labs, 1998a).

Evidence from In Vivo Studies of Monkeys

Two studies have evaluated biomarkers of PPARα activation in monkeys. Oral (gavage) exposure to DINP had no effect on PBOX in male cynomolgus monkeys treated with 500 mg/kg-day DINP for 14-days (Pugh et al., 2000). Similarly, no effect on cyanide-insensitive palmitoyl CoA oxidase activity or cytochrome P450 concentration and lauric acid 11- and 12-hydroxylase activities in hepatic microsomes were observed in male and female marmosets gavaged with up to 2,500 mg/kg-day DINP for 13 weeks (Hall et al., 1999).

4.1.2 Key Event 2: Alterations in Cell Growth Pathways

EPA identified one *in vivo* study of mice investigating alterations in cell growth pathways. No *in vivo* studies of rats or monkeys for KE2 were identified. Ma et al. (2014a) administered DINP via oral gavage to male Kunming mice at 0, 0.2, 2, 20, and 200 mg/kg-day DINP daily for 14 days and then determined TNFα and IL-1 in liver homogenates. IL-1 and TNFα content was significantly increased at 20 and 200 mg/kg-day. However, this study is limited by the fact that study authors do not identify the specific IL-1 subtypes evaluated (*e.g.,* IL-1α vs. IL-1β).

4.1.3 Key Event 3: Perturbation of Cell Growth and Survival

Evidence of increased cell proliferation comes from five *in vivo* studies of mice, two *in vivo* studies of rats, one *in vivo* study of monkeys, and two in *vitro* studies of primary rat and human hepatocytes. Across *in vivo* studies of mice and rats, an acute cell proliferative response in the liver is consistently observed. In contrast, cellular proliferation in the liver is not sustained chronically in either species. However, as discussed by Corton et al. (2018), PPARα activators tend to "produce transient increases in replicative DNA synthesis during the first few days or weeks of exposure followed by a return to baseline levels." Therefore, lack of a sustained proliferative response is consistent with the proposed MOA. No evidence of replicative DNA synthesis was observed in one *in vivo* study of monkeys. In the two *in vitro* studies, DINP consistently suppressed apoptosis and increased replicative DNA synthesis in rat, but not human hepatocytes. Available data for KE3 is discussed further below.

Evidence from In Vitro Studies

Two *in vitro* studies are available that consistently demonstrate that DINP can suppress apoptosis and increase replicative DNA synthesis in rat but not human hepatocytes. Hasmall et al. (1999) treated primary rat hepatocytes obtained from male F344 rats and primary human hepatocytes with 250 to 750 µM DINP. Treatment with DINP increased replicative DNA synthesis, suppressed apoptosis, and suppressed TGFβ1-induced apoptosis in rat but not human hepatocytes. Similarly, Shaw et al. (2002)

treated primary rat hepatocytes obtained from male F344 rats and primary human hepatocytes with 150 to 250 µM DINP and observed treatment-related suppression of apoptosis and increased replicative DNA synthesis in rat but not human hepatocytes.

Evidence from In Vivo Studies of Rats

Two studies of rats have evaluated cell proliferation in the liver following oral exposure to DINP. In both studies, bromodeoxyuridine (BrdU) was administered to rats via osmotic minipumps and cell proliferation was evaluated via BrdU labeling. No *in vivo* studies of rats have evaluated effects on hepatocyte apoptosis. Smith et al. (2000) reported treatment-related increases in hepatocellular replicative DNA synthesis in male F344 rats fed diets containing 12,000 ppm DINP (approximately 1,200 mg/kg-day) for 2 or 4 weeks; however, no change was observed in the low-dose group (approximately 100 mg/kg-day). In the second study, increased hepatocellular replicative DNA synthesis was observed in male and female F344 rats after 1 week of dietary exposure to 733 (males) or 885 (females) mg/kg-day DINP, but not after 2, 13, or 104 weeks of exposure (Covance Labs, 1998b).

Evidence from In Vivo Studies of Mice

Five studies have evaluated cell proliferation (measured via BrdU labeling in all five studies) and/or apoptosis in the liver following oral exposure to DINP. Valles et al. (2003) fed female B6C3F1, SV129, and *Pparα*-null mice diets containing 8,000 ppm DINP (approximately 1,200 mg/kg-day) for 1 week and observed increased hepatocellular replicative DNA synthesis in B6C3F1 and SV129 mice, but not *Pparα*-null mice. Smith et al. (2000) report treatment-related increases in hepatocellular replicative DNA synthesis in male B6C3F1 mice fed diets containing up to 6,000 ppm DINP (approximately 900 mg/kg-day) for 2 but not 4 weeks. Further, no change in replicative DNA synthesis was observed in the low-dose group at either timepoint (approximately 75 mg/kg-day). Two other studies reported no increase in hepatocellular replicative DNA synthesis in the livers of male or female B6C3F1 mice dosed with 2,600 mg/kg-day DINP for 4, 31, and 91 days (Hazleton Labs, 1992) or 1,560 (males) to 1,888 (females) mg/kg-day DINP for 79 and 105 weeks (Covance Labs, 1998a).

In another study, Kaufmann et al. (2002) evaluated hepatocellular replicative DNA synthesis and apoptosis (via TUNEL staining) in male and female B6C3F1 mice administered 117 to 2,806 mg/kg-day DINP for 1 or 4 weeks. Dose-related increases in hepatocellular replicative DNA synthesis were observed in male and female mice after 1 week at doses as low as 116 (male) to 1,272 (female) mg/kg-day; however, no significant changes in females were noted after 4 weeks at doses as high as 2,806 mg/kg-day, while significant increases in males after 4 weeks were observed at doses as low as 117 mg/kg-day but without a clear dose-response relationship. In males, apoptosis was increased after 1 week in the high-dose group (1,860 mg/kg-day). At 4 weeks, apoptosis appeared reduced in all treatment groups for males; however, the effect was not statistically significant. No clear treatment-related effects on apoptosis were observed for females at either timepoint.

Evidence from In Vivo Studies of Monkeys

Treatment with DINP had no effect on replicative DNA synthesis (measured via proliferating cell nuclear antigen [PCNA] immunohistochemistry) in male cynomolgus monkeys treated with 500 mg/kg-day DINP for 14 days (Pugh et al., 2000).

4.1.4 Key Event 4: Selective Clonal Expansion of Preneoplastic Foci

EPA identified no *in vitro* or *in vivo* studies of DINP that evaluated KE4. Further, hepatocellular hyperplasia, which may provide some evidence of expansion of preneoplastic foci, has not been reported in any short-term, subchronic, or chronic studies of DINP.

4.1.5 Modulating Factors

EPA identified no studies evaluating activation of NF-κB in the liver.

Two studies provide data on the relationship between oxidative stress and DINP following *in vivo* exposures in male Kunming mice (Ma et al., 2014b) or *in vitro* investigations in human hepatic cell-types (Gutiérrez-García et al., 2019). Available studies provide evidence that DINP can induce ROS in the liver.

Ma et al. (2014b) exposed male Kunming mice to DINP via oral gavage daily for 14 days and evaluated several endpoints related to oxidative stress in homogenized hepatic tissue. Indices of oxidative stress were generally observed at the same doses that resulted in histopathological lesions of the liver, although quantification of the tissue sections was not performed. Dose-dependent increases in ROS and increases in malondialdehyde were observed, reaching significance at 200 mg/kg-day. In parallel, decreases in glutathione content occurred at 200 mg/kg-day DINP, indicative of oxidative stress. The authors also reported DNA-protein-crosslinks and increases in 8-hydroxydeoxyguanosine at 200 mg/kg-day, which indicate oxidative damage to DNA.

An *in vitro* study in HepG2 cells by Gutiérrez-García et al. (2019) evaluated the potential for DINP to elicit oxidative stress and investigated a mechanism involving sirtuins (srts), which are a group of mitochondrial NAD+-dependent histone deacetylases. Increases in ROS were observed at the highest concentration tested in parallel with increases in lysine acetylation and dose-dependent reductions in expression of several sirtuin genes (*i.e., Sirt1*, *Sirt2*, *Sirt3*, *Sirt5*), as well as decreases in sirtuin protein levels. Although the data does not directly provide evidence that ROS is a modulating factor within the PPARα activation MOA for hepatic tumors, considered more broadly, it does suggest that DINP can induce ROS in hepatocytes.

4.2 Dose-Response Concordance of Key Events with Tumor Response

Dose-Response Concordance: Rats

As discussed in Sections 4.1.1 through 4.1.4, data from *in vivo* rat studies is limited to KE1, KE3, and the apical outcome, hepatocellular adenomas and/or carcinomas. No data is available for KE2 or KE4. Available data used by EPA for its dose-response concordance analysis of the PPARα MOA in rats is presented in Table 4-1.

Although limited, there is some evidence to demonstrate that KE1 occurs at lower doses than KE2 and the apical outcome, liver tumors. For KE1, three studies report consistent dose-related increases in several biomarkers of PPARα activation (*i.e.,* increased PBOX, lauric acid 11- and 12-hydroxylase, palmitoyl-CoA oxidase activity) (Smith et al., 2000; Covance Labs, 1998b; BIBRA, 1986). The lowest dose at which PPARα activation was reported in rats is 442 mg/kg-day, following 104 weeks of exposure to DINP (Covance Labs, 1998b). For KE3, one study reports a dose-related increased in hepatocellular replicative DNA synthesis at very high doses of DINP (*i.e.,* 1,200 mg/kg-day) after 2 and 4 weeks of exposure (Smith et al., 2000). A second study, which only evaluated hepatocellular replicative DNA synthesis at a single dose (*i.e.,* 733 (males) to 885 (females) mg/kg-day), reports increased hepatocellular replicative DNA synthesis and palmitoyl-CoA oxidase activity after 1 week of exposure (Covance Labs, 1998b). Statistically significant dose-related increases in hepatocellular carcinomas and/or combined adenomas and carcinomas have been observed in two studies of rats at doses at low as 672 to 885 mg/kg-day (Covance Labs, 1998b; Bio/dynamics, 1987). In the study of F344 rats by Covance Labs (1998b), increased hepatic palmitoyl-CoA oxidase activity (KE1) was observed in female (but not male) rats at lower doses than which adenomas and carcinomas were observed after 104 weeks of treatment (*i.e.,* 442 vs. 885 mg/kg-day for tumors), providing evidence of concordance.

Overall, there is some evidence to support dose-response concordance for KE1, KE3, and hepatocellular adenomas and/or carcinomas. However, no data are available for KE2 or KE4, or apoptosis (part of KE3) in rat hepatocytes, which prevents a complete analysis of dose-response concordance across all KEs in the postulated MOA.

Dose-Response Concordance: Mice

As discussed in Sections 4.1.1 through 4.1.4, data from *in vivo* mouse studies is limited to KE1, KE2, KE3, and the apical outcome, hepatocellular adenomas and/or carcinomas. No data is available for KE 4. Available data considered by EPA for its dose-response concordance analysis of the PPARα MOA in mice is presented in Table 4-2.

Although limited, available data indicate the KE1, KE2, and KE3 occur in mice at lower doses than hepatocellular adenomas and/or carcinomas, providing some evidence of concordance. However, concordance across KE1, KE2, and KE3 is less apparent. As can be seen from Table 4-2, the lowest dose at which biomarkers of PPARα activation were increased was 117 mg/kg-day for male mice after 4 weeks of exposure (Kaufmann et al., 2002); for KE2 increased TNFα and IL-1 in liver homogenate has been observed at doses as low as 20 mg/kg-day (Ma et al., 2014a); for KE3 increased DNA synthesis has been reported at doses as low as 116 mg/kg-day in male mice (Kaufmann et al., 2002); and hepatocellular adenomas and carcinomas have been observed at doses as low as 336 mg/kg-day in female mice. However, there are several sources of uncertainty related to KE2 data from Ma et al. (2014a). First, Ma et al. evaluated DINP exposure with Kunming mice, while other studies of DINP were performed with B6C3F1 mice, and it is unclear if there is a strain difference in sensitivity or if studies testing lower doses of DINP with B6C3F1 mice would produce similar results. Additionally, Ma et al. report increased IL-1 in liver homogenate, but do not differentiate between cytokine subtypes (*e.g.,* IL-1α, IL-1β). Another limitation of the available dataset is that PBOX is generally not considered as sensitive of a biomarker as other measures of PPARα activation, especially compared to measures of PPARα-inducible genes.

844 **Table 4-1. Dose-Response Concordance for PPARα MOA in Rats**

Dose (mg/kg-day)	KE 1 (Sex; Dose in mg/kg-day; Timepoint)	KE 2	KE3 (Sex; Dose in mg/kg-day; Timepoint)	KE 4	Hepatocellular Tumors
1–200	NC – PBOX (M; 120; 2, 4 wks)[a]	–	NC – DNA synthesis (M; 120; 2, 4 weeks)[a]	–	NC – Neoplastic nodules, hepatocellular cancer, or combined (M/F; 15–184; 104 weeks)[d] NC – Adenomas, carcinomas, combined (M/F, 29–109; 104 weeks)[b] NC – Neoplastic nodules, carcinoma (M/F; 27–33; 104 weeks)[e]
201–400	NC – PP (M/F; 307-375; 2 yrs)[d]	–	–	–	NC – Neoplastic nodules, hepatocellular cancer, or combined (M/F; 307–375; 104 weeks)[d] NC – Adenomas, carcinomas, combined (M/F, 359-442; 104 weeks)[b] NC – Neoplastic nodules, carcinoma (M/F; 271–331; 104 weeks)[e]
401–600	↑ Palm CoA (F (not M); 442; 104 (but not 1,2, or 13) wks)[b]	–	–	–	–
601–1,000	NC – Palm CoA (M/F;607–639; 3 weeks)[c] ↑ 11/12 H-lase (M, not F); 639; 3 weeks)[c] ↑ Palm CoA (M/F; 733–885; 1, 2, 13, 104 weeks)[b]	–	↑ DNA synthesis (M/F; 733–885; 1 week (but not 2, 13, 104 weeks)[b]	–	↑ Carcinoma (F (not M); 672; 104 weeks)[e] ↑ Carcinoma (M (not F); 733–885; 104 weeks)[b] ↑ Combined adenoma and carcinoma (M/F); 733–885; 104 weeks)[b]
1,001–1,400	↑ Palm CoA (M/F; 1,192–1,198; 3 weeks)[c] ↑ 11/12 H-lase (M, not F); 1,192; 3 weeks)[c] ↑ PBOX (M; 1,200; 2, 4 weeks)[a]	–	↑ DNA synthesis (M; 1,200; 2,4 weeks)[a]	–	–
1,401–2,000		–	–	–	–
2,001–2500	↑ 11/12 H-lase (M/F; 2,195–2,289; 3 weeks)[c]	–	–	–	–

[a] (Smith et al., 2000)
[b] (Covance Labs, 1998b)
[c] (BIBRA, 1986)
[d] (Lington et al., 1997)
[e] (Bio/dynamics, 1987)
11/12 H-lase = lauric acid 11- and 12-hydroxylase; F = female; M = male; NC = no significant change; Palm CoA: cyanide-insensitive palmitoyl-CoA oxidation; PBOX = peroxisomal beta-oxidation; PP = peroxisomal proliferation
'-' indicates no experimental evidence is available

845

Table 4-2. Dose-Response Concordance for PPARα MOA in Mice

Dose (mg/kg-day)	KE 1 (Sex; Dose in mg/kg-day; Timepoint)	KE 2	KE3 (Sex; dose in mg/kg-day; timepoint)	KE 4	Hepatocellular Tumors
1–200	NC – PBOX (M; 75; 2,4 weeks)[a] ↑ PP & Palm CoA (M (but not F); 117; 4 weeks)[b]	NC – TNFα (M, 0.2–2, 2 weeks)[f] ↑ TNFα (M, 20–200, 2 weeks)[f]	NC – DNA synthesis (M; 75; 2, 4 weeks)[a] ↑ DNA synthesis (M (but not F); 116-167; 1, 4 weeks)[b] NC – Apoptosis (M/F; 116-167; 1, 4 weeks)[b]	–	NC – Adenomas or carcinomas (M/F; 90–112, 2 yrs)[d]
201–400	↑ PP & Palm CoA (M; 350; 4 weeks)[b] ↑ Palm CoA (M/F; 365; 4, 31, 91 days)[c]	–	↑ DNA synthesis (M; 337-350; 1, 4 weeks)[b] NC – Apoptosis (M; 337-350; 1, 4 weeks)[b]	–	↑ Combined adenomas & carcinomas (F (but not M); 336, 2 yrs)[d]
401–600	↑ PP & Palm CoA (F; 546; 4 weeks)[b] ↑ Palm CoA (M/F; 600; 2 weeks)[e]	–	NC – DNA synthesis (F; 520-546; 1, 4 weeks)[b] NC – Apoptosis (F; 520-546; 1, 4 weeks)[b]	–	–
601–800	–	–	–	–	↑ Combined adenomas & carcinomas (M; 742, 2 yrs)[d]
801–1,000	↑ PBOX (M; 900; 2,4 weeks)[a] ↑ PP & Palm CoA (M; 913; 4 weeks)[b]	–	↑ DNA synthesis (M; 75; 2 (not 4) weeks)[a] ↑ DNA synthesis (M; 901-913; 1, 4 weeks)[b] NC – Apoptosis (M; 901-913; 1, 4 weeks)[b]	–	↑ Carcinomas and combined adenomas & carcinomas (F; 910, 2 yrs)[d]
1,001–1,400	↑ Palm CoA (M/F; 1,200; 2 weeks)[e] ↑ PP & Palm CoA (F; 1,272; 4 weeks)[b]	–	↑ DNA synthesis (F; 1200; 1 week)[e] ↑ DNA synthesis (F; 1272-1278; 1 (but not 4) weeks)[b] NC – Apoptosis (F; 1272-1278; 1, 4 weeks)[b]	–	–
1,401–2,000	↑ PP & Palm CoA (M; 1860; 4 wks)[b] ↑ Palm CoA (M/F; 1,560–1,888; 79, 105 wks)[d]	–	↑ DNA synthesis (M; 1766-1860; 1, 4 weeks)[b] NC – DNA synthesis (M/F; 1,560–1,888; 79, 105 weeks)[d] ↑ Apoptosis (M; 1,766–1,860; 1 (but not 4) weeks)[b]	–	↑ Adenomas and/or carcinomas (M/F; 1,560–1,888, 2 yrs)[d]
2,001–3,000	↑ Palm CoA (M/F; 2600; 4, 31, 91 days)[c] ↑ PP & Palm CoA (F; 2806; 4 weeks)[b]	–	↑ DNA synthesis (F; 2593-2806; 1 (but not 4) weeks)[b] NC – DNA synthesis (M/F; 2,600; 4, 41, 91 days)[c] NC – Apoptosis (F; 2,593–2,806; 1, 4 weeks)[b]	–	–

[a] (Smith et al., 2000)
[b] (Kaufmann et al., 2002)
[c] (Hazleton Labs, 1992)
[d] (Covance Labs, 1998a)
[e] (Valles et al., 2003)
[f] (Ma et al., 2014a)
↑ = significant increase; ↓ = significant decrease; 11/12 H-lase = lauric acid 11- and 12-hydroxylase; F = female; M = male; NC = no significant change; Palm CoA: cyanide-insensitive palmitoyl-CoA oxidation; PBOX = peroxisomal beta-oxidation; PP = peroxisomal proliferation
'-' indicates no experimental evidence is available

4.3 Temporal Association of Key Events with Tumor Response

In rats, it is clear that KE1 and KE3 precede tumor formation, however, the temporal sequence of KE1 and KE3 cannot be established (Table 4-1). Biomarkers of PPARα activation (KE1) and hepatic cell proliferation (KE3) are both increased as early as 1 week following oral exposure to DINP (Covance Labs, 1998b); however, no studies are available that evaluate either KE at early timepoints. Comparatively, liver neoplasms were first detected during an interim sacrifice on study week 79 in a study of F344 rats by Covance Labs (1998b) (albeit without a clear dose-relationship; adenomas detected in one control male and one high-dose female; carcinoma detected in one high-dose male).

In mice, it is clear that KE1, KE2, and KE3 precede tumor formation; however, the temporal sequence of KE1, KE2, and KE3 cannot be established (Table 4-2). Biomarkers of PPARα activation (KE1) are significantly increased in one study as early as 4 days after oral exposure (Hazleton Labs, 1992), while KE2 is measured in only a single study that reports increases in TNFα and IL-1 in liver homogenate after 14 days (Ma et al., 2014a), and hepatic cell proliferation (KE3) is increased after 1 week of oral exposure to DINP (Kaufmann et al., 2002). However, no studies are available that evaluate any of these KEs at earlier timepoints. Comparatively, in the available 2-year bioassay of mice (Covance Labs, 1998a), hepatocellular adenomas and carcinomas were first detected on study days 167 and 366, respectively, in a single high-dose male at each timepoint (as reported by (U.S. CPSC, 2001)).

4.4 Strength, Consistency, and Specificity of Association of Tumor Response with Key Events

Available *in vivo* studies of mice and rats and *in vitro* studies of rat and mouse hepatocytes provide remarkably consistent evidence that DINP can activate PPARα (KE1).There is also consistent evidence that DINP can cause acute proliferative cellular responses in the livers of rats and mice *in vivo* and rat hepatocytes *in vitro* (KE3). In contrast, cellular proliferation in the liver is not sustained chronically in either species. As discussed by Corton et al. (2018), PPARα activators tend to "produce transient increases in replicative DNA synthesis during the first few days or weeks of exposure followed by a return to baseline levels." Chronic or sustained proliferative responses for potent PPARα activators tend to be much lower compared to acute proliferative responses. Comparatively, DINP is a relatively weak PPARα activator and low levels of chronic hepatic cell proliferation may be difficult to detect over variable background levels. Therefore, lack of a detectable sustained proliferative response is consistent with the proposed MOA for a weak PPARα activator such as DINP. Further adding to the strength of evidence, KE1 and KE3 have been observed in studies of differing design and originating from different laboratories, with hepatic effects such as increases in relative liver weight and hepatocellular hypertrophy observed in short-term, subchronic, and chronic studies of rats and mice. These effects, although not KEs in the PPARα MOA, are frequently observed following PPARα activation and subsequent peroxisome proliferation.

A notable inconsistency in the database stems from an unexplained difference in sensitivity across sexes in mice. In the 2-year bioassay of mice, liver tumors were observed at doses as low as 335 mg/kg-day in female mice and 742 mg/kg-day in male mice (Covance Labs, 1998a), indicating female mice are more sensitive than males. In contrast, other studies have demonstrated that PPARα activation (KE1) and cellular proliferation (KE3) occur at lower doses in male mice compared to females (Kaufmann et al., 2002). This apparent inconsistency cannot be explained.

4.5 Biological Plausibility and Coherence

Extensive evidence exists to support the hypothesis that chronic PPARα activation can lead to alterations in cell growth pathways, perturbations of cell growth and survival, and selective clonal expansion of preneoplastic foci cells leading to hepatocellular tumorigenesis in rodents (reviewed in (Corton et al., 2018; Corton et al., 2014)). This proposed MOA for DINP-induced liver tumors in rats and mice is consistent with available data, indicating biological plausibility. Available data from mice and rats demonstrate PPARα activation after short-term (several days to weeks) oral exposure to DINP that can be sustained with chronic exposure (Covance Labs, 1998a, b). Although studies also demonstrate that oral exposure to DINP can cause acute hepatic cell proliferative responses, other studies demonstrate that oral exposure to DINP does not cause chronic proliferative response in the liver of mice or rats. As discussed by Corton et al. (2018) chronic or sustained proliferative responses for potent PPARα activator are much lower compared to acute proliferative responses. Comparatively, DINP is a relatively weak PPARα activator and low levels of chronic hepatic cell proliferation may be difficult to detect over variable background levels.

4.6 Other Modes of Carcinogenic Action

This section summarizes evidence for other modes if carcinogenic action in the liver for DINP.

Ppara-Null Mice

Valles et al. (2003) conducted a series of short-term (1- to 3-week) studies in which male and female B6C3F1, wild-type SV129, and *Ppara*-null mice were exposed to DINP. Repeated dose studies well-established that in response to exposure to DINP, male and female B6C3F1 wild-type show hepatotoxicity. Across these studies, dose-dependent increases in relative liver weight that were dependent on PPARα were generally observed; however, in one study of older (30-week) female *Ppara*-null mice, PPARα-independent increases in relative liver weight has also been observed, (these increases were specific for older female mice; younger female or older male *Ppara*-null mice did not exhibit any changes in liver to body weight ratios after exposure to DINP), thereby hinting at the possibility of PPARα-independent mechanisms being at play in the liver under certain conditions. Unique gene expression changes in older *Ppara*-null female mice have been identified in expression arrays, like testosterone hydroxylase (Cyp2d9). Cyp2d9 is down-regulated by DINP in wild-type mice, but Cyp2d9 was up-regulated in *Ppara*-null mice. The relevance of these subtle PPARα-independent effects to hepatocarcinogenesis is not known, but *Ppara*-null mice are resistant to the carcinogenicity of a prototypical PPARα activator (Peters et al., 1997). It is important to note that most of the studies conducted by Valles et al. support the hypothesis that PPARα plays a dominant role in mediating the carcinogenic effects of DINP in the liver.

Other Nuclear Receptors

Constitutive androstane receptor (CAR), pregnane X receptor (PXR), and aryl hydrocarbon receptor (AhR) are known to play a role in liver homeostasis and disease. Although their precise role, if any, in liver tumorigenesis in response to chronic exposure to DINP has not yet been established. In addition to PPARα, DINP has been shown to activate multiple nuclear receptors that may play a role in liver tumorigenesis. Several studies have demonstrated that DINP can activate CAR, which is a nuclear receptor with an adverse outcome pathway with KEs like those of PPARα and has been implicated in hepatic carcinogenesis in rodents (Felter et al., 2018). DeKeyser et al. (2011) used transactivation and mammalian two-hybrid assays in COS-1 cells to demonstrate that DINP is a strong activator of human CAR variant 2 (hCAR2). Furthermore, DINP induced expression of CYP2B6, one of the primary target genes of CAR, in primary human hepatocytes. In a subsequent study by the same research group, Laurenzana et al. demonstrates that MINP, metabolite of DINP, can also activate hCAR2 (Laurenzana et

al., 2016). Additionally, *in vitro* studies have also shown that DINP /MINP can activate human PXR (Laurenzana et al., 2016; Dekeyser et al., 2011) as well as mouse and human PPAR gamma, although the degree of PPAR gamma activation was greater for the mouse receptor than for the human receptor under the conditions of the study (Bility et al., 2004). DINP has also been shown to promote and induce tumorigenesis in a variety of cell types through AhR-mediated genomic and nongenomic pathways (Wang et al., 2012). DINP induces several changes in rodent liver consistent with PPARα activation (Laurenzana et al., 2016). DINP induces some of these liver changes independently of PPARα activation as shown in *Pparα*-null mice (Valles et al., 2003).

DINP has also been evaluated in 442 high-throughput assays as part of EPA's Toxicity ForeCaster (ToxCast) program. Curated high-throughput screening data for DINP accessed through the National Toxicology Program's Integrated Chemical Environment (ICE) indicated that DINP was inactive in the majority of tested assays and active in only seven assays (Table 4-3). Consistent with available literature, DINP was active in two assays for PXR activation. However, DINP was inactive in assays for other nuclear receptors (*i.e.,* CAR, AhR, PPARα, PPARγ) and other assays of PXR (*i.e.,* TOX21_PXR_Agonist, TOX21_PXR_viability) and these results are inconsistent with available literature.

Table 4-3. Summary of Active ToxCast Assays for DINP[a]

ToxCast Assay	Mode of Action	AC50/LOEC (µM)
BSK_SAg_Eselectin_up	Cancer - KCC6: Chronic Inflammation, CardioTox – Endothelial Injury/Coagulation	0.2
BSK_CASM3C_TissueFactor_down	AcuteTox – Immune and Inflammatory Response, CardioTox – Endothelial Injury/Coagulation	0.2
ATG_PXRE_CIS_up	Cancer – KCC8: Receptor Mediated Effects	1.2
ATG_PXR_TRANS_up	Cancer – KCC8: Receptor Mediated Effects	1.7
BSK_KF3CT_IL1a_down		4
NVS_ENZ_hBACE		8.7
ACEA_ER_AUC_viability	AcuteTox - Cytotoxicity, Cancer – KCC10: Cell Proliferation/Death/Energetics	38.8

AC50 = concentration at which 50% maximum activity is observed; LOEC = lowest-observed-effect-concentration
[a] Data accessed through NTP's Integrated Chemical Environment in February 2024.

Gap Junction Intercellular Communication

Gap junctional intercellular communication (GJIC) is the only portal by which multicellular organisms mediate the intercellular exchange of cellular signal factors from the interior of one cell to that of neighboring cells (Loewenstein, 1987; Pitts and Finbow, 1986). GJIC is considered to play a crucial role in the maintenance of homeostasis, and in turn, aberrant GJIC is likely to be involved in carcinogenesis, given that cancer cells do indeed behave as if they have dysfunctional GJIC and are dissociated from the homeostasis maintained by the organism. Inhibition of GJIC has been proposed as a non-genotoxic carcinogenic mechanism (Yamasaki et al., 1995; Yamasaki, 1995). Aberrant GJIC has been known as a non-genotoxic event that is important for carcinogenesis. This is based on the observation that many non-genotoxic tumor-promoting agents inhibit GJIC (Klaunig et al., 2003). Several tumor types, including hepatocellular carcinomas, have been shown to demonstrate inhibited GJIC (Trosko et al.,

1990c; Trosko et al., 1990a, b; Trosko and Chang, 1989). DINP is shown to inhibit hepatic GJIC, and the inhibition of GJIC has been proposed as a non-genotoxic carcinogenic mechanism, in rodents exposed to DINP for 2 or 4 weeks (Smith et al., 2000; Trosko et al., 1990c; Trosko et al., 1990b).

Cytotoxicity and Regenerative Proliferation

Cytotoxicity followed by regenerative proliferation is an established nongenotoxic MOA (Felter et al., 2018). There is some limited evidence that DINP may act through a cytotoxic MOA. The KEs for establishing a cytotoxic MOA are (1) the chemical is not DNA reactive; (2) evidence of cytotoxicity by histopathology (*e.g.,* the presence of necrosis and/or increased apoptosis); (3) evidence of toxicity by increased serum enzymes indicative of cellular damage that are relevant to humans; (4) presence of increased cell proliferation as evidenced by increased labeling index and/or increased number of hepatocytes; (5) demonstration of a parallel dose response for cytotoxicity and formation of tumors; and (6) reversibility upon cessation of exposure (Felter et al., 2018). As discussed in Section 2 as well as below in the genotoxicity section, EPA considers DINP not likely to be genotoxic or mutagenic. Four studies have provided quantitative liver histopathology with clear evidence of lesions consistent with cytotoxicity, namely focal necrosis, including three 2-year bioassay studies in rats (Covance Labs, 1998b; Lington et al., 1997; Bio/dynamics, 1986), one 13-week study in mice (Hazleton Labs, 1992), and one 4-week study in mice (Hazleton Labs, 1991). In Lington et al (1997), a significant dose-related increased incidence of focal necrosis was observed in male rats, and the Bio/dynamics study (1987) reported increased incidence of focal necrosis in males of the mid-dose group, with no clear dose-response. In the rat study by Covance Labs (1998b), individual cell degeneration/necrosis was significantly increased in males of the high-dose group. However, not all chronic studies reported this lesion. The 2-year study in mice by Covance Labs (1998a) did not observe focal necrosis or apoptosis, even with a study design that included higher doses.

As mentioned above in Section 4.1.3, DINP has been shown to elicit acute proliferative responses in mouse hepatocytes *in vivo* and *in vitro*. Hyperplasia has not been observed in hepatic tissues, suggesting against regenerative proliferation. Increases in periportal hepatocellular replicative DNA synthesis have been reported in mice and rats following exposure to 12,000 ppm DINP for 2 or 4 weeks (Smith et al., 2000), consistent with increases in hepatocyte proliferation observed in two other mouse studies at doses ranging from 150 to 8,000 ppm for 1 to 4 weeks (Valles et al., 2003; Kaufmann et al., 2002) or in rats up to 855 mg/kg-day DINP for up to 104 weeks (Covance Labs, 1998b). Two *in vitro* studies (Shaw et al., 2002; Hasmall et al., 1999) reported increased replicative DNA synthesis and suppressed apoptosis in rat hepatocytes at doses of DINP ranging from 150 to 750 µM. The available data do not consistently support the various KEs in the MOA for cytotoxicity, suggesting other MOAs are at play.

4.7 Uncertainties and Limitations

There are several limitations and uncertainties associated with the available dataset for the postulated PPARα MOA. First, no data is available for KE2 and KE4 for rats or mice, with the exception of a single study of mice that reported increased TNFα and IL-1 (KE2) in liver homogenate (Ma et al., 2014a). However, that study is limited in that it evaluated a single duration of exposure (14 days) and did not distinguish between IL-1 subtypes (*i.e.,* IL-1α, IL-1β). Lack of data for KE2 and KE4 is a data gap, which reduces EPA's confidence in the postulated PPARα MOA.

For KE3, only one *in vivo* study of mice (and none of rats) is available that examined apoptosis in the liver (Kaufmann et al., 2002). In the available study, apoptosis was significantly increased after one week of exposure to DINP and was unaffected after 4 weeks. This is inconsistent with the postulated MOA, in which suppression of apoptosis is anticipated. However, this uncertainty is somewhat addressed by the two available *in vitro* studies of rat hepatocytes that report consistent, dose-related,

increases in PPARα activation (KE1), increases in replicative DNA synthesis (KE3) and suppression of apoptosis (KE3) in hepatocytes following exposure to DINP (Shaw et al., 2002; Hasmall et al., 1999).

Most of the available data for KE1 and KE3 comes from *in vivo* studies of rats and mice; however, available studies are of variable design and in some instances employ large dose spacing, which makes comparisons across studies difficult. Although it is clear that KE1 and KE2 occur at lower doses and earlier than the apical outcome, liver tumors, providing some evidence of dose-response and temporal concordance, concordance between KEs could not be established, which reduces EPA's confidence in the postulated PPARα MOA.

Another uncertainty stems from an unexplained difference in sensitivity across sexes in B6C3F1 mice. In the 2-year bioassay of B6C3F1 mice, liver tumors were observed at doses as low as 335 mg/kg-day in female mice and 742 mg/kg-day in male mice (Covance Labs, 1998a). In contrast, other studies have demonstrated that PPARα activation and proliferative DNA synthesis occur at lower doses in male B6C3F1 mice compared to females (Kaufmann et al., 2002). This inconsistency further reduced EPA's confidence in the postulated PPARα MOA.

Despite remaining uncertainties, there is strong evidence to support the postulated PPARα MOA. Available evidence indicates that DINP is not genotoxic (Section 2). Furthermore, other potential modes of carcinogenic action, such as activation of CAR, PXR, and AhR, as well as cytotoxicity and regenerative proliferation are also non-genotoxic threshold MOAs. Finally, as discussed further below in Section 4.8, the chronic non-cancer point of departure (POD) identified in EPA's *Draft Non-cancer Human Health Hazard Assessment for Diisononyl Phthalate (DINP)* (U.S. EPA, 2024) will adequately account for all chronic toxicity, including carcinogenicity and activation of PPARα (KE1), which could potentially result from exposure to DINP.

4.8 Weight of Scientific Evidence: Cancer Classification

Under the *Guidelines for Carcinogen Risk Assessment* (U.S. EPA, 2005), EPA reviewed the weight of evidence and determined that DINP is *Not Likely to be Carcinogenic to Humans* at doses below levels that do not result in PPARα activation (KE1). This classification was based on the following weight of scientific evidence considerations:

- DINP exposure resulted in treatment related PPARα activation (KE1) in male mice at doses greater than or equal to 117 mg/kg-day (Kaufmann et al., 2002) and female rats at doses greater than or equal to 442 mg/kg-day (Covance Labs, 1998b).
- DINP exposure resulted in treatment related liver tumors (adenomas and/or carcinomas combined) in female mice at doses greater than or equal to 336 mg/kg-day DINP (Covance Labs, 1998a) and female rats at doses greater than or equal to 672 mg/kg-day DINP (Bio/dynamics, 1987).
- Available MOA data for liver tumors in mice and rats support the proposed PPARα MOA.
- Limited data are available that indicate a role for other non-genotoxic, threshold, MOAs, including activation of other nuclear receptors (*e.g.*, CAR, PXR, AhR, PPARγ), inhibition of GJIC, and cytotoxicity and regenerative proliferation.
- There is no evidence for mutagenicity.

Further, the non-cancer chronic POD (NOAEL/LOAEL of 15/152 mg/kg-day based on non-cancer liver effects (see *Draft Non-cancer Human Health Hazard Assessment for Diisononyl Phthalate (DINP)* (U.S. EPA, 2024)) will adequately account for all chronic toxicity, including carcinogenicity, which could potentially result from exposure to DINP. In one study of male mice (Kaufmann et al., 2002), biomarkers of PPARα activation were significantly increased at 117 mg/kg-day, which is less than the

chronic LOAEL of 152 mg/kg-day based on non-cancer liver effects. Although, the study by Kaufman et al. did not test sufficiently low doses to establish a NOAEL for PPARα activation, other studies of mice have established a NOAEL of 75 mg/kg-day for PPARα activation (Smith et al., 2000). Therefore, the non-cancer chronic POD of 15 mg/kg-day is considered protective of PPARα activation.

4.9 Human Relevancy

Several panels have been convened to address the human relevancy of liver tumors in rodents occuring through a PPARα MOA (Felter et al., 2018; Corton et al., 2014). These panels have generally concluded that the PPARα MOA is not relevant to humans or unlikely to be relevant to humans based on qualitative and quantitative differences between species. Nevertheless, uncertainty and differing scientific opinions on the human relevance of the PPARα MOA for liver tumorigenesis remain, despite the related efforts of previous panels and workshops.

Several authoritative agencies have evaluated the role of PPARα and peroxisome proliferation in inducing hepatocellular tumors in rodents following chronic exposure to DINP. Australia NICNAS (2012) and U.S. CPSC (2010) concluded that liver tumors in rodents observed following exposure to DINP are not likely to be human relevant, while ECHA (2013) and Health Canada (EC/HC, 2015) concluded that liver tumors in rats are of unclear human relevance. However, none of these agencies quantitatively evaluated DINP for carcinogenic risk to humans.

As discussed further in EPA's *Draft Non-cancer Human Health Hazard Assessment for Diisononyl Phthalate (DINP)* (U.S. EPA, 2024), not all of the non-cancer liver effects observed in rodents are consistent with PPARα activation (*e.g.,* spongiosis hepatis). Furthermore, the non-cancer chronic POD (NOAEL/LOAEL of 15/152 mg/kg-day) that is based on non-cancer liver toxicity will adequately account for all chronic toxicity, including carcinogenicity, which could potentially result from exposure to DINP.

5 CONCLUSIONS AND NEXT STEPS

DINP has been evaluated for carcinogenicity in two 2-year dietary studies of F344 rats (Covance Labs, 1998b; Lington et al., 1997), one 2-year dietary study of SD rats (Bio/dynamics, 1987), and one 2-year dietary study of B6C3F1 mice (Covance Labs, 1998a). Across available studies, treatment-related hepatocellular adenomas and carcinomas have consistently been observed in F344 and SD rats as well as B6C3F1 mice. Existing assessments of DINP by U.S. CPSC (2014, 2010), Health Canada (ECCC/HC, 2020; EC/HC, 2015; Health Canada, 2015), ECHA (2013), and NICNAS (2012) have postulated that DINP causes liver tumors in rats and mice through a PPARα MOA. Consistent with *EPA Guidelines for Carcinogen Risk Assessment* (U.S. EPA, 2005) and the IPCS *Mode of Action Framework* (IPCS, 2007), EPA further evaluated the postulated PPARα MOA for liver tumors, as well as evidence for other plausible MOAs for DINP.

Although some uncertainties remain, there is strong evidence to support the postulated, non-genotoxic, PPARα MOA. Under the *Guidelines for Carcinogen Risk Assessment* (U.S. EPA, 2005), EPA determined that DINP is *Not Likely to be Carcinogenic to Humans* at doses below levels that do not result in PPARα activation (KE1). Further, the non-cancer chronic POD (NOAEL/LOAEL of 15/152 mg/kg-day based on non-cancer liver effects; see EPA's *Draft Non-cancer Human Health Hazard Assessment for Diisononyl Phthalate (DINP)* (U.S. EPA, 2024)) will adequately account for all chronic toxicity, including carcinogenicity, which could potentially result from exposure to DINP. Therefore, the non-cancer chronic POD of 15 mg/kg-day is considered protective of PPARα activation and carcinogenicity.

EPA is soliciting comments from the Science Advisory Committee on Chemicals (SACC) on charge questions and comments from the public for an upcoming SACC meeting.

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Appendix A PATHOLOGY WORKING GROUP REVIEW FOR SPONGIOSIS HEPATIS AND MNCL (EPL, 1999)

A Histopathology Peer Review and a Pathology Working Group (PWG) review (EPL, 1999) was conducted on selected lesions of the liver and spleen observed in F344 rats in the 2-year bioassays reported by Lington et al. (1997) and Covance Labs (1998b). The PWG review evaluated the significance of spongiosis hepatis, foci of cellular alteration, primary hepatocellular neoplasms in the liver, and the significance of MNCL. The peer and PWG reviews were conducted in accordance with EPA Pesticide Regulation Notice 94-5 that describes the procedure to be followed for submission of pathology re-reads to the Agency (EPL, 1999).

Spongiosis Hepatis

Induction of spongiosis hepatis, also referred to as cystic degeneration by some authors, is of interest because it appears to be the most sensitive non-neoplastic response in rats chronically exposed to DINP (Covance Labs, 1998b; Lington et al., 1997). However, questions have arisen regarding the relationship of this lesion to other pathological processes occurring in animals treated with DINP that may not be relevant to humans, including peroxisome proliferation and MNCL. Although a few differences were noted, the Histology Peer Review and the PWG review of lesions in the liver and spleen generally confirm the incidence data reported by the original study pathologists. The incidences of spongiosis hepatis in the Lington et al. (1997) and Covance Labs (1998b) studies as determined by the PWG are shown in Table_Apx A-1 and Table_Apx A-2.

The PWG noted that spongiosis hepatis might be found as an independent lesion or within foci of cellular alteration or hepatocellular neoplasms. In the reviewed studies, spongiosis hepatis was diagnosed whenever it occurred, regardless of relationship to other hepatic changes that were also present. This method of diagnosis differs from some standard pathology guidelines, which recommend that spongiosis hepatis not be diagnosed separately when it occurs within foci or tumors. The PWG concluded that the method of diagnosis used in the DINP rat studies made interpretation of spongiosis hepatis as a treatment-related effect difficult. As noted in EPL (1999), some differences were noted in the pathology protocols for the two studies which may have affected the reported incidences. These differences include the number of sections taken from the liver in each study and the protocol for examination of the spleen. These differences make the direct comparison of the results from Lington et al. (1997) and Covance Labs (1998b) difficult and may account for the greater incidence of foci of cellular alteration and foci of spongiosis hepatis observed by Lington et al. (1997).

Table_Apx A-1. Incidence of MNCL and Selected Hepatic Lesions at Terminal Sacrifice (104 Weeks) in the Lington et al. (1997) Study in F344 Rats as Determined by the PWG (EPL, 1999)

Lesion	Dose Group mg/kg-day (ppm)			
	Control	15 M / 18 F (300)	152 M / 184 F (3,000)	307 M / 375 F (6,000)
Males				
MNCL	32/81	27/80	48/80	49/80
Hepatocellular adenoma	3/81	1/80	2/80	1/80
Hepatocellular carcinoma	0/81	1/80	0/80	3/80
Eosinophilic foci	58/81	50/80	46/80	52/80
Basophilic foci	53/81	62/80	48/80	42/80
Spongiosis hepatis	22/81	24/80	51/80	62/80
Females				
MNCL	22/81	21/81	29/80	41/80
Hepatocellular adenoma	0/81	4/81	0/80	2/80
Hepatocellular carcinoma	1/81	0/81	0/80	1/80
Eosinophilic foci	59/81	47/81	42/80	32/80
Basophilic foci	72/81	64/81	64/80	55/80
Spongiosis hepatis	4/81	1/81	3/80	4/80

Source: Modified from data in Table 6 in EPL (1999)
M = male; F = female

Table_Apx A-2. Incidence of MNCL and Selected Hepatic Lesions at Terminal Sacrifice (104 Weeks) in the Covance Labs (1998b) Study in F344 Rats as Determined by the PWG (EPL, 1999)

Lesion	Dose Group mg/kg-day (ppm)					
	Control	29 M / 36 F (500)	88 M / 109 F (1,500)	359 M / 442 F (6,000)	733 M / 885 F (12,000)	Recovery 637 M / 773 F (12,000)
Males						
MNCL	21/55	23/50	21/50	32/55	28/55	30/50
Hepatocellular adenoma	2/55	4/50	1/50	4/55	7/55	6/50
Hepatocellular carcinoma	1/55	0/50	0/50	3/55	11/55	3/50
Eosinophilic foci	22/55	14/50	16/50	15/55	10/55	12/50
Basophilic foci	40/55	34/50	33/50	28/55	27/55	25/50
Spongiosis hepatis	6/55	6/50	3/50	18/55	26/55	10/50
Females						

Lesion	Dose Group mg/kg-day (ppm)					
	Control	29 M / 36 F (500)	88 M / 109 F (1,500)	359 M / 442 F (6,000)	733 M / 885 F (12,000)	Recovery 637 M / 773 F (12,000)
MNCL	17/55	16/50	9/50	28/55	28/55	24/50
Hepatocellular adenoma	1/55	1/50	0/50	1/55	1/55	1/50
Hepatocellular carcinoma	0/55	0/50	0/50	1/55	6/55	2/50
Eosinophilic foci	10/55	5/50	7/50	7/55	0/55	4/50
Basophilic foci	37/55	32/50	31/50	18/55	5/55	13/50
Spongiosis hepatis	0/55	0/50	0/50	1/55	2/55	0/50

Source: Modified from data in Tables 9 and 10 in EPL (EPL, 1999)
M = male; F = female

Examination of Co-occurrence of MNCL and Spongiosis Hepatis

It has been suggested that the occurrence of spongiosis hepatis in rats exposed to DINP is a consequence of MNCL (EPL, 1999). To address this possibility, the PWG examined the co-occurrence of spongiosis hepatis and MNCL in the study by Lington et al. (1997) and Covance Labs (1998b). A comparison of the numbers of animals with spongiosis hepatis with and without MNCL diagnosed by the study pathologist did not support the conclusion that spongiosis hepatis is a consequence of MNCL as shown in Table_Apx A-3. Although approximately half of the rats with spongiosis hepatis also had MNCL, spongiosis hepatis was also observed in the absence of MNCL in the remainder of the affected animals.

Table_Apx A-3. Comparison of Spongiosis Hepatis with MNCL as Determined by the PWG (EPL, 1999)

Sex	Dose Group (ppm)	Total with Spongiosis Hepatis	Spongiosis Hepatis without MNCL	Spongiosis Hepatis with MNCL
Comparison of data from Lington et al. (1997)				
F	0	4	1	3
F	300	1	1	0
F	3,000	3	0	3
F	6,000	4	1	3
M	0	24	16	8
M	300	24	12	12
M	3,000	54	17	37
M	6,000	66	27	39
Comparison of data from Covance Labs (1998b)				
F	0	0	0	0
F	500	0	0	0
F	1,500	0	0	0
F	6,000	1	0	1

Sex	Dose Group (ppm)	Total with Spongiosis Hepatis	Spongiosis Hepatis without MNCL	Spongiosis Hepatis with MNCL
F	12,000	2	0	2
F	12,000 recovery	0	0	0
M	0	5	1	4
M	500	5	4	1
M	1,500	2	1	1
M	6,000	14	8	6
M	12,000	21	11	10
M	12,000 recovery	9	5	4

Source: Modified from data in Tables 11 and 12 in EPL (1999)
M = male; F = female

1447